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Osteonecrosis of the Jaws: Investigations into Pathophysiology and Prevention

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Oral Biology

by

Danny Hadaya

2020

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ABSTRACT OF THE DISSERTATION

Osteonecrosis of the Jaws: Investigations into

Pathophysiology and Prevention

by

Danny Hadaya

Doctoral of Philosophy in Oral Biology

University of California, Los Angeles

Professor Sotirios Tetradis, Chair

Osteonecrosis of the Jaws (ONJ), a significant side effect of anti-resorptive or anti-angiogenic medications, is described as exposed bone in the oral cavity that often presents with pain, infection, and often significantly decreases quality of life of affected patients. Largely seen in patients taking anti-resorptives for osteoporosis, bone malignancies, and bone metastases, the pathophysiology of ONJ has remained largely elusive. Additionally, treatment options for the disease have remained empirical, often focused on managing symptomology. Others have recommended surgical resection of the affected region, often leaving large defects that can be devastating. Here, we develop an ONJ animal model in rats, harness its power to investigate the effects of anti-resorptive discontinuation. Additionally, we explore whether other medications with possible anti-resorptive properties can lead to development of ONJ. Finally, we evaluate clinical data to better understand ONJ pathophysiology, as well as ONJ treatment.

Following clinical observations, where tooth extraction occurs only in diseased teeth, such as those with periapical or periodontal disease, we hypothesized that significant periapical disease prior to tooth extraction will lead to ONJ. Thus, we treated rats with high-dose bisphosphonates, and extracted teeth with experimental periapical disease (EPD). All BP-naïve animals healed uneventfully. Interestingly, only animals treated with BPs with extraction of teeth with EPD developed clinical, radiographic, and histologic signs of ONJ. Our findings here show that pre-existing periapical disease is necessary to induce ONJ in rats treated with BPs.

Using the animal model described above, we investigated with discontinuation of BPs or the RANK-L inhibitor, OPG-Fc (a denosumab surrogate for rodents), is time or drug dependent. First, we observed that discontinuation of BPs prior to tooth extraction did not ameliorate ONJ burden, while discontinuation of OPG-Fc prior to tooth extraction ameliorated ONJ burden. In contrast, discontinuation of BPs or OPG-Fc in rats with established ONJ did not lead to resolution. These findings have direct clinical correlation and can be used for the development of clinical guidelines.

With our understanding of ONJ and its relationship to systemic anti-resorptive use, we investigated whether treatment with romosozumab, a monoclonal antibody against sclerostin used for the treatment of osteoporosis, would lead to ONJ. In clinical studies, romosozumab led to decreased bone resorption markers; thus, while not a purely catabolic agent, it may have the potential to cause ONJ. Using an established model of experimental periodontitis (EP) in ovariectomized rats, we found that treatment with romosozumab did not lead to ONJ.

Driven by these translational findings, we evaluated a cohort of patients with osteopetrosis and pycnodysostosis, inherited genetic diseases that lead to an anti-resorptive like phenotype. In these patients, we observed areas of exposed, non-healing bone that were

phenotypically identically to patients with ONJ from anti-resorptive use. Radiographic and histologic findings confirmed findings; in-vitro resorption pit assays revealed an absence of osteoclastic resorptive activity in these patients.

Finally, we retrospectively evaluated a cohort of ONJ patients that had been treated with local wound care. In these patients, we found that over 75% of patients healed with bone sequestration, leaving healed mucosa on the underlying surface. We found that healing time correlated with their wound care abilities, with patients with better wound care exfoliating the necrotic bone prior to those with poor wound care.

The dissertation of Danny Hadaya is approved.

Maie St. John

Paul Krebsbach

Tara Aghaloo

Sotirios Tetradis, Committee Chair

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2020

DEDICATION

This work is dedicated to

my family, friends and mentors

who have taught me more about life than I could ever imagine

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LIST OF ACRONYMS

AAOMS= American Association of Oral and Maxillofacial Surgeons

ANOVA= One-way analysis of variance

ASBMR= American Society for Bone and Mineral Research

ATP= Adenosine triphosphate

BP= Bisphosphonate

BRONJ= Bisphosphonate Related Osteonecrosis of the Jaws

BV/TV= Bone Volume / Tissue Volume

CBCT= Cone Beam Computed Tomography

CEJ= Cementoenamel Junction

DMARD= Disease Modifying Anti-Rheumatic Drug

EDTA= Ethylenediaminetetraacetic acid

EP= Experimental Periodontitis

EPD= Experimental Periapical Disease

IP= Intraperitoneal

IV= Intravenous

GTP= Guanine triphosphate

M-CSF= Macrophage Colony-Stimulating Factor

ONJ= Medication Related Osteonecrosis of the Jaws

ONJ = Osteonecrosis of the Jaws

OPG= Osteoprotegrin

ORONJ= Osteoclast Related Osteonecrosis of the Jaws

OVX= Ovariectomy

P1NP= Procollagen Type 1 N-terminal propeptide

PBMC= Peripheral Blood Mononuclear Cell

PE= Pulp Exposure

RANK= Receptor of activator Nuclear-factor Kappa-beta

RANK-L = Receptor of activator Nuclear-factor Kappa-beta Ligand

ROI= Region of Interest

Scl-Ab= Sclerostin-antibody

SEM= Standard Error of the Mean

ZA= Zoledronic Acid

µCT= Micro-computed Tomography

Veh= Vehicle

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analysis, interpretation and experimental design. Tetradis S served as the lead PI and was involved in all aspects of the research.

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Publications

- Gkouveris I, **Hadaya D**, Soundia A, Bezouglaia O, Chau Y, Dry SM, Pirih FQ, Aghaloo T, Tetradis S. "Vasculature Submucosal Changes at Early Stages of Osteonecrosis of the Jaw (ONJ)*".*
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CHAPTER 1: INTRODUCTION

History and Definition

Osteonecrosis of the Jaws (ONJ) is defined as exposed bone in the oral cavity that lasts for at least 8 weeks, in patients with a history of anti-resorptive or anti-angiogenic use, and no history of head and neck radiation (1,2). ONJ was first described in a letter to the editor in 2003 and subsequently in a case series in 2004, as refractory osteomyelitis in patients taking intravenous bisphosphonates (BPs) or oral BPs (3,4).

A task force set forth by the American Association of Oral and Maxillofacial Surgeons (AAOMS) in 2007 initially described the disease in patients taking BPs, potent inhibitors of osteoclast function (5). Thus, the disease was identified as Bisphosphonate Related Osteonecrosis of the Jaws (BRONJ). In 2014, an update was provided by the AAOMS, renaming the disease to ONJ due to the inclusion of denosumab, and anti-angiogenics, as other medications which can cause ONJ (2). A new anti-resorptive agent in 2010, denosumab, is a monoclonal antibody against Receptor Activator of Nuclear-factor Kappa-B Ligand (RANK-L), which prevents osteoclast differentiation and maturation (6,7). The new class of medications, anti-angiogenics inhibit the creation of blood vessels, include medications such as tyrosine kinase inhibitors (8). Since 2014, ONJ has also been described in patients taking other medications, chemotherapeutics and disease modifying anti-rheumatic drugs (DMARDs) (9-11). It is likely that these medications cause a similar phenotypic appearance, but have different pathophysiologic processes.

Background

Anti-resorptive agents include BPs, originally described decades ago, and more recently, denosumab, a monoclonal antibody against RANK-L (6,7,12). These medications are

commonly prescribed for patients with osteoporosis, multiple myeloma, Paget's disease, and hypercalcemia of malignancy (13-15). These medications are also prescribed for patients with bone metastases; most commonly due to breast cancer in women, and prostate cancer in men(12). The overall goal use of these medications is aimed at preservation of bone structure and limiting skeletal related events (SREs), such as a pathologic bone fractures.

BPs are classified into two types, nitrogen and non-nitrogen containing (12). Notwithstanding the type, BPs resemble inorganic pyrophosphate, and very strongly bind to hydroxyapatite in bone, where they are incorporated into areas of active remodeling (12). Once deposited into areas of bone remodeling, they are taken up by the osteoclast. In the early non-nitrogen containing BPs, such as clondronate or etidronate, the BPs would be incorporated into molecules of adenosine triphosphate (ATP) (15). This new ATP cannot be released through hydrolysis, leading to its accumulation. Finally, due to the lack of hydrolyzable ATP, cell processes are inhibited, ultimately leading to osteoclast apoptosis. This osteoclast apoptosis deems the osteoclast dysfunctional, leading to inhibition of bone resorption.

Newer nitrogen-containing BPs, such as alendronate, commonly prescribed for osteoporosis, and zoledronic acid, often given to patients with malignancies, are much more potent than their earlier counterparts. Nitrogen-containing BPs, once uptaken by an osteoclast, inhibit an important enzyme necessary for the production of cholesterol (16). This enzyme, farnesyl pyrophosphate synthase, is important in the synthesis of farnesyl pyrophosphate and geranylgeranylphyrophosphate, molecules that are important for post translational modification of small Rho GTPases (17). Rho GTPases are necessary for osteoclast activity, and their inhibition ultimately leads to osteoclast apoptosis.

On the other hand, denosumab, a monoclonal antibody against RANKL, prevents osteoclast differentiation and maturation by affecting the RANKL/RANK/osteoprotegrin (OPG) pathway (7). This pathway is important for regulation of bone homeostasis, where RANK-L binds to the RANK receptor on early, non-differentiated osteoclasts (18). Once bound, this leads to osteoclast formation. OPG, on the other hand, acts as a decoy receptor for RANKL, binding any circulating RANKL, and preventing osteoclast differentiation and maturation (18). Similar to OPG, denosumab binds to circulating RANKL, preventing osteoclast differentiation, and thus, function.

While both BPs and denosumab function to inhibit osteoclast function, there are important distinctions among the two. BPs can be administered orally, or intravenously; however, their bioavailability is poor when administered orally (6). Less than 1% of the available BP is absorbed after an oral dose. Although the half-life of BPs remains largely unknown, it has previously been shown excreted in urine 10 years following a single intravenous dose (19). Additionally, BPs that are not incorporated into the remodeling skeleton are secreted via the kidneys. Denosumab is a monoclonal antibody, which has a half-life of around 29 days, much less than the previously described BPs (20). Importantly, denosumab is not incorporated into the skeletal system and is cleared via the mononuclear phagocyte system shortly after administration (21). Thus, it's elimination from the body is well described.

Prevalence & Incidence

Anti-resorptives prescriptions can be divided into two categories: those that receive high-doses for treatment of an underlying malignancy or bone metastases, and those who receive low doses for treatment of osteoporosis. Current literature estimates that the prevalence of ONJ in

patients taking low dose anti-resorptives, such as weekly alendronate, for osteoporosis is around 0.001-0.01%, an extremely low number (1). In a survey of patients in Ontario, Oral and Maxillofacial Surgeons identified 13 patients who received oral bisphosphonates and developed ONJ, a 0.001% cumulative incidence rate over three years (22). In a randomized group of 240 men, where half received denosumab for osteoporosis, no cases of adjudicated ONJ were reported (23). In a group of 4,550 osteoporotic women, who received denosumab for three or six years, six cases of ONJ were identified (24). It must be noted that it is extremely difficult to assess the validity of these numbers due to the low incidence of ONJ; however, it is clear that the risk of ONJ in patients with osteoporosis is low.

In contrast, in patients taking high-dose anti-resorptives, the incidence has been estimated anywhere from 1-5%, with some studies achieving the upper limit of the number. In a clinical trial comparing BPs and denosumab for the treatment of bone metastases in breast cancer patient, ONJ occurred in 1.4% in BP treated patients and in 2.0% of patients treated with denosumab (25). In a prospective study following 80 patients treated with high-dose BPs for multiple myeloma or other malignancies, 28% development ONJ (26). In males treated with BPs or denosumab for prostate cancer, ONJ occurred in 1% and 2% of the patients, respectively (27). The incidence rates in large clinical trials provide early outcomes for ONJ; however, it is likely that these numbers increase over longer periods of time.

Staging

ONJ has been classically defined into three stages, based largely on clinical and radiographic findings (2). In stage one, patients are asymptomatic and present with exposed bone with an absence of infection. Stage two patients are defined as those with exposed bone that is symptomatic and evidence of infection. This can be seen as erythema, edema, or purulent

discharge. Finally, stage three patients are those who present with one of the following: 1) pathologic fracture of the mandible, 2) oroantral or oronasal communication, 3) extra-oral fistula, 4) exposed bone that involves more than the alveolar bone, or 5) those with osteolysis that extends past the maxillary sinus or inferior border of the mandible.

A more controversial topic has been the inclusion of a stage "zero" ONJ. This is known as a non-exposed variant of ONJ. Stage 0 ONJ presents with an absence of exposed bone, however, non-specific symptoms can be present, such as dull pain, or erythema, or edema. Radiographic signs, such as osteosclerosis, periosteal bone formation, or sequestration can aid in the diagnosis. Stage 0 can be diagnosed when there is no odontogenic source of the aforementioned non-specific symptoms. However, concern must be taken to avoid over diagnosis of the disease. Published studies have identified that around 50% of patients diagnosed with stage 0 ONJ ultimately develop bone exposure (28,29). A recent study by Soundia et al. identified that patients with more significant radiographic features, such as cortical erosion, sequestration, and periosteal reaction are likely to progress to bone exposure (28).

Pathophysiology

While much has been learned about ONJ, some questions remain. Tissue level data have provided insights into disease pathophysiology, however, molecular level remains scant, and our understanding of underlying molecular changes that lead to the development of exposed bone remain scant. Current hypotheses include bone remodeling suppression, direct cytotoxicity of drugs on oral tissues, immune dysfunction, infection and inflammation, and angiogenesis inhibition.

Anti-resorptives are known to suppress bone resorption in areas of active remodeling. BPs directly lead to osteoclast apoptosis, while denosumab prevents their formation by affecting

the OPG/RANK/RANKL access(6). Additional data to support this hypothesis was the discovery of ONJ in patients taking denosumab (30). As denosumab also targets osteoclast function, it is more likely that the effects of BPs on bony remodeling is critical in ONJ pathogenesis. Interestingly, the prevalence of ONJ in patients on BPs and denosumab is similar, supporting the notion that suppression of bone remodeling is critical in ONJ pathogenesis (1,2). It is interesting, however, that the side of ONJ is localized to the oral cavity. In 2014, Cheong et al. evaluated the localization of anti-resorptive using a fluorescently label BP (31). Their findings showed that BPs accumulated preferentially in areas of tooth extraction, or in areas with periapical inflammation.

It has also been hypothesized that BPs have a direct cytotoxic effect on fibroblasts, osteoblasts, and other cells present in the oral cavity. Walter et al. evaluated the effects of nitrogen and non-nitrogen containing BPs on fibroblasts and osteoblasts (32). In-vitro, these BPs induce apoptosis, decrease cell viability and migration (32). Additionally, in 2009, Scheper and others evaluated how Zoledronic Acid (ZA) affects oral fibroblasts and epithelial cells (33). They found that ZA induces cell death, and propose that this cell death and lack of cellular growth leads to bone exposure, and ultimately ONJ (33). A manuscript published by Taniguchi et al. compared the effects of BPs on human periodontal ligament fibroblasts vs. dermal fibroblasts (34). Interestingly, they found that BPs cause released of reactive oxygen species in oral fibroblasts, but not dermal fibroblasts (34). While there is in-vitro evidence to suggest that BPs in high concentrations can lead to apoptosis of fibroblasts and other mucosal cells, this hypothesis became less likely when the first report of denosumab, a different anti-resorptive, was associated with ONJ(30).

Initial reports of ONJ occurred following tooth extraction (4). What is sometimes forgotten is that teeth are often extracted due to periapical or periodontal disease, both

inflammatory processes that are known to occur in the presence of periapical or periodontal pathogens. Interestingly, it is often difficult to induce ONJ in animal models following tooth extraction unless pre-existing inflammation exists (35-38). In a study by Dimopoulos, the risk of ONJ was decreased by completing preventative measures prior to initiating treatment with bisphosphonates in patients with multiple myeloma or other tumors (39). In a study by Mawardi et al., tooth extraction in mice followed by infection with *Fusobacterium nucleatum* in mice treated with pamidronate led to development of ONJ-like lesions (40). A study of 38 patients identified that total bacterial level in patients with ONJ, compared with non-ONJ controls (41). Together, these results point to an important role of bacteria and inflammation in the pathogenesis of ONJ.

Another hypothesis for ONJ development is an interruption of vascular supply to the affected areas. This initially has been driven due to the phenomenon of avascular osteonecrosis of the hip, which is due to a loss of blood supply (42). When damaged due to traumatic events occurs, the lateral and medical circumflex arteries, which provide blood supply to the femoral head, can be damaged, leading to avascular osteonecrosis (43). In the setting of bisphosphonate therapy, Ziebart et al., evaluated the effects of bisphosphonate use on endothelial progenitor cells, finding that BPs led to cell apoptosis (44). Additionally, Fournier and others reported that BPs inhibit angiogenesis in vitro, and in-vivo, showing a 50% decrease in revascularization of the prostate (45). Gkouveris et al. identified a decrease in the arterial and venous network surrounding areas of developing ONJ (46). Together, these data point to angiogenesis inhibition as a factor in ONJ development.

Treatment Strategies

ONJ, since its initial appearance in literature has been difficult to treat. In general, evaluation of treatment options is difficult in this diverse patient population because many authors have different criteria for disease resolution. This can include mucosalization, decrease in symptoms, decrease in staging, decrease in infection, decrease in exposed bone area, and other clinical measurements that can be made. There have generally been two treatment approaches described, a non-surgical and surgical approach.

Initial treatment focused on non-surgical management of symptomology, with little attempt at full healing. This becomes exceedingly important in patients who are medically compromised, or those who have significantly limiting systemic disease with poor prognoses (47). Initial treatment options for these individuals were aimed at increasing quality of life, and pain management. This non-surgical treatment focused on the use of antibiotic rinses, in conjunction with systemic antibiotic treatment in those with significant soft tissue infection; Lazarovici and colleagues used long-term antibiotics in an attempt to treat ONJ; however, had little success, with only 18% of patients achieving full disease resolution (48). Non-surgical treatment is the focus of treatment strategies in patients with stage 0, 1, or 2 ONJ, in accordance with AAOMS guidelines.

Surgical treatment is reserved for those with refractory disease, and those that present with pathologic fractures. Recently, this approach has become increasing popular, and has shown promise in its use (49,50). This often involves marginal or segmental resection of the affected maxilla or mandible to an area of "bleeding bone", which can often be difficult to reconstruct. However, some patients may not be able to tolerate these surgical procedures due to their underlying medical conditions. Despite these limitations, surgical intervention can be an option

for those who require immediate relief and are unable to manage symptoms. Interestingly, Carlson et al. have identified metastatic lesions in the jaws following surgical resection (51).

Rationale and Hypotheses

Osteonecrosis of the Jaws, since it's description almost 20 years ago, has remained exceedingly difficult to treat, with treatment options designed to manage symptoms, or others designed to surgically remove large portions of the jaws. This devastating disease continues to affect individuals taking anti-resorptive medications for both osteoporosis, those with malignant diseases, and those with bone metastases. Our common goal here was to both understand underlying disease pathophysiology, and to investigate possible treatment options for this devastating disease.

A common appearance in most ONJ cases appears to be tooth extraction, in conjunction with systemic anti-resorptive therapy. However, only 1-5% of patients develop ONJ. Thus, our rationale was to evaluate the role of dental inflammation in ONJ development, in the form of periapical disease. Others have described that removal of pre-existing inflammation can ameliorate ONJ development (52). Using a rodent model, we induced periapical infection in the molars, and then further exacerbated the condition by inoculating the pulp chamber with known periapical pathogens. To evaluate whether ONJ would develop, we then extracted these teeth, and evaluated extraction sockets clinically, radiographically, and histologically.

Interestingly, other medications also seem to decrease osteoclast function. In patients treated with systemic romosozumab, a monoclonal antibody against sclerostin (Scl-Ab), bone resorption markers were decreased (53-55). Thus, it is plausible that this decrease in osteoclast function can also lead to ONJ. Here, we chose to use an ONJ model of experimental periodontitis in ovariectomized rats to test whether animals treated with Scl-Ab develop ONJ. Following systemic treatment with Veh, ZA, or Scl-Ab and development of EP, we evaluated radiographic and histologic signs of ONJ.

In our studies, we observed that inflammation and infection are strongly associated with ONJ development. We hypothesized that removal of the inflammation and infection around ONJ lesions can lead to disease resolution. Therefore, we evaluated the effects of local wound care on ONJ resolution in a cohort of over 100 patients.

Both literature and our studies have identified that inhibition of bone resorption is crucial to the development of ONJ. Interestingly, the genetic conditions, osteopetrosis and pycnodysostosis, lead to an anti-resorptive like phenotype. These genetic conditions disrupt osteoclast function, similar to BPs and denosumab. We evaluated clinical, radiographic, and histologic findings in a group of patients who presented with areas of exposed bone, with the hypothesis that they suffer from a genetically induced form of ONJ.

One potential therapeutic option for ONJ development is sometimes termed a “drug holiday”. Due to the different pharmacologic properties of BPs and denosumab, we hypothesized that discontinuation of denosumab, but not BPs, prior to tooth extraction can ameliorate ONJ development. We also hypothesized that discontinuation of neither BPs nor denosumab, in patients with developed ONJ will lead to disease resolution. We tested these hypotheses using a rat model of ONJ, and evaluated clinical, radiographic, and histologic findings.

Specific Aims

Specific Aim 1: To evaluate whether extraction of teeth with experimental periapical disease in rats treated with high-dose anti-resorptives leads to the development of ONJ.

- The effects of experimental periapical disease were evaluated using µCT imaging and histology.
- Following evaluation of experimental periapical disease, socket healing in rats treated with high-dose BPs was assessed clinically, radiographically, and histologically.

Specific Aim 2: To investigate if antibodies against sclerostin, a negative regulator of bone formation with both osteoanabolic and catabolic effects, can lead to the development of ONJ with periodontal disease.

- Ovariectomy was used to mimic post-menopausal osteoporosis, after which rats were systemically treated with sclerostin antibodies and high-dose BPs.
- A model of ligature induced periodontitis was used to evaluate development of ONJ using clinical, radiographic, and histologic signs.

Specific Aim 3: To test whether local aggressive wound care in patients with existing ONJ can lead to disease resolution.

- Patient charts were retrospectively analyzed for their wound care score.
- Wound care score was correlated to time to disease resolution.
- Patient demographics were assessed for correlation with disease resolution.

Specific Aim 4: To compare the exposed bone in patients with osteopetrosis and pycnodysostosis to those treated with BPs and denosumab.

- Clinical, radiographic, and histologic assessment was made of exposed bone in patients with osteopetrosis and pycnodysostosis.
- A comparison of the exposed bone in patients with genetic diseases to those with ONJ due to anti-resorptives was made.

Specific Aim 5: To evaluate the effects of anti-resorptive discontinuation before and after tooth extraction.

- Clinical, radiographic, and histologic evaluation of tooth extraction in rats where BP and OPG-Fc discontinuation occurred prior to tooth extraction.
- Clinical, radiographic, and histologic assessment of ONJ amelioration following discontinuation of BP or OPG-Fc in rats with ONJ.

CHAPTER 2: Development of Medication Related Osteonecrosis of the Jaw (ONJ) after extraction of teeth with experimental periapical disease

ABSTRACT

Objectives: Medication Related Osteonecrosis of the jaw (MRONJ) is a rare, but severe side effect of antiresorptive medications. Most animal models utilize tooth extraction as an instigating local factor to induce ONJ, with varied results. However, these teeth are healthy, absent of dental disease, a rare finding that does not reflect clinical practices. We hypothesize that extraction of teeth with periapical inflammation leads to ONJ in rats treated with high-dose bisphosphonates.

Materials and Methods: Rats were pre-treated with Zoledronic Acid (ZA) for 1-week. Pulp Exposure (PE) was established by exposing the pulpal chamber of the first and second molars. Experimental Periapical Disease (EPD) was induced by pulp exposure and bacterial inoculation into pulp chambers of the first and second mandibular molars. The mandibular molars were extracted 4-weeks following PE or EPD, and animals euthanized 4-weeks after tooth extraction. Extraction sockets were assessed clinically, radiographically, and histologically.

Results: Clinically, radiographically and histologically, socket healing was observed in all Veh animals, and in ZA animals after extraction of healthy teeth or teeth with PE. In contrast, bone exposure, lack of socket healing and osteonecrosis were present in the majority of the ZA animals after extraction of teeth with EPD. Bacterial presence was noted in areas of osteonecrotic alveolar bone.

Conclusion: Our data support a synergistic contribution of severe dental disease and tooth extraction for ONJ pathogenesis. Importantly, this model is amenable to manipulation of methodological conditions for the dissection of parameters involved in MRONJ pathogenesis.

INTRODUCTION

Anti-resorptive medications, such as bisphosphonates and denosumab, prescribed for management of bone malignancy or osteoporosis (56,57), have rare, but serious side effects, including Medication Related Osteonecrosis of the Jaw (MRONJ). MRONJ is characterized by exposed bone in the maxillofacial region for at least 8 weeks, without a history of head and neck radiation (2). The most common local risk factor for MRONJ development is tooth extraction (58), which in the majority of adult patients is caused by periodontal or periapical disease (59). While prevalence is low, MRONJ decreases quality of life and can lead to serious complications (1,60). Even though MRONJ has been studied extensively, its pathophysiology remains elusive (1,3). A variety of mechanisms have been proposed, including osteoclast dysfunction, bone turnover suppression and altered wound healing (61-64). Animal models that reproduce some, but not all, aspects of human MRONJ have been developed, utilizing two main approaches: tooth extraction or development of periapical or periodontal disease (65,66).

In the first approach, animals treated with antiresorptives undergo tooth extraction. However, unlike in humans, the extracted teeth in these models are healthy and void of periapical or periodontal disease. This is a rare occurrence in the clinical management of adult patients, as teeth are often extracted due to severe periodontal or periapical disease. Interestingly, several studies utilizing extraction of healthy teeth in animals under high dose antiresorptive treatment report defective osseous, but normal mucosal healing (65,67-71). Mucosal defects are more consistently observed in animals on antiresorptives also treated with adjunctive therapies, such as steroids or chemotherapy, or during vitamin D deficiency or diabetes (69-72). These adjuvant treatments likely compromise soft and hard tissue healing, and have a significant contribution to MRONJ development. On the other hand, others have reported defective mucosal healing in

animals treated only with antiresorptive treatment (73-75). However, there are technical differences in these studies that likely account for the disparate observations.

In the second approach toward development of MRONJ animal models, periapical or periodontal disease is induced in animals treated with antiresorptives, but no tooth extraction is performed (62,63,76,77). Such models report clinical, radiographic and histologic observations that resemble features of MRONJ. As MRONJ largely occurs following tooth extraction, the insight provided by these models is limited only to cases of spontaneous MRONJ around teeth with periodontal or periapical disease. Despite the limitations, these models provide insight into disease pathogenesis.

Here, we propose that combining the two approaches would more closely capture the clinical reality of MRONJ pathogenesis. We hypothesize that extraction of teeth with periapical inflammation, but not of healthy teeth, leads to MRONJ in rats treated with high-dose BPs.

MATERIALS AND METHODS

Animal care

Eight-week old, healthy, male Wistar-Han rats (Charles River Laboratories, Raleigh, NC) were randomly assigned to receive intraperitoneal (IP) injections of endotoxin-free saline (vehicle) or 200μg/kg Zoledronic Acid (ZA) (LKT Laboratories, St Paul, MN) twice weekly, in morning hours (219-261g, 236g average). Vehicle (Veh) or ZA treatment was continued throughout the duration of the experiments. Throughout the experiment, rats were housed in pathogen-free conditions (2 per cage) with a 12-hour light/dark cycle, fed a standard diet (NIH-31 Modified Open Formula, ENVIGO, Madison, WI) and given water *ad libitum*. Rats with retained root fragments or fractured cortical plates were excluded from analysis. All applicable institutional and/or national guidelines for the care and use of animals were followed.

Experiment 1: Extraction of teeth with Pulp Exposure (PE)

Following 1 week of pre-treatment, 24 rats (12 Veh, 12 ZA) had the crowns of their right first (M1) and second (M2) mandibular molars drilled using a 1/2 round carbide burr to create pulpal exposure. 4 weeks after pulpal exposure, the right M1 and M2 were extracted. Animals were euthanized 4 weeks following tooth extraction. 2 Veh and 2 ZA animals were excluded from analysis due to retained root fragments/fractured cortical plates.

Experiment 2: Effects of bacterial inoculation on periapical disease

After 1 week of pre-treatment, 40 rats (20 Veh, 20 ZA) had the right M1 and M2 drilled to create pulpal exposure, as described. In 10 ZA and 10 Veh treated animals, the pulpal chambers were inoculated with a solution of periapical pathogens containing 10^9 of each

Porphyromonas gingivalis, *Streptococcus gordonii*, *Aggregatibacter actinomycetemcomitans*, and *Fusobacterium nucleatum* to induce experimental periapical disease (EPD). The pulpal chamber was covered with Cavit (3M ESPE, St. Paul, MN). 8 weeks after EPD, animals were euthanized.

Experiment 3: Extraction of teeth with Experimental Periapical Disease (EPD)

After 1 week of pre-treatment, EPD, described above, was induced in the right M1 and M2 of 24 Veh and 36 ZA treated animals. 24 Veh and 22 ZA treated animals, serving as controls, did not undergo any manipulation prior to extraction. 4 weeks following induction of EPD, all animals had M1 and M2 extracted. Animals were euthanized 4 weeks following tooth extraction. 4 Veh and 5 ZA animals with extraction of healthy teeth, and 8 Veh and 12 ZA animals with EPD were excluded from analysis due to retained root fragments.

Ex-vivo µCT specimen scanning & Imaging

Mandibles were harvested, then imaged using a digital microscope at 40x magnification (Keyonce VHX-1000, Osaka, Japan). Following fixation in 4% paraformaldehyde for 48 hours, Mandibles were imaged by ex-vivo µCT using SkyScan 1172 at 20 µm resolution (SkyScan, Kontich, Belgium), as described (66). Volumetric data were converted to DICOM format and imported to generate reconstructed images. Linear and volumetric measurements of periapical bone loss, and bone volume fractionation (BV/TV) were made, as described (62). Healing of extraction sockets was rated as complete (greater than 75% of the socket), partial (healing of 25-75% of the socket) or absent (less than 25% of the socket), and quantified, as described (37).

Histology, TRAP staining, gram staining

Mandibles were decalcified in 15% EDTA and sectioned in a buccal-lingual fashion, in the area of bone exposure. Samples were paraffin embedded and 5μm sections were made and stained with H&E (38). Analysis was performed using Aperio Image Scope software (Aperio Technologies, Inc., Vista, CA). The region of interest (ROI) was defined as the area of the alveolar crest to the inferior border of the mandible in the area of M1 and M2. The epithelium to alveolar crest distance, total number of osteocytic lacunae, number of empty lacunae, and osteonecrotic area were quantified (38). Empty lacunae were those with empty or karolytic osteocytic lacunae. Osteonecrosis were identified as an area of 5 or more confluent empty lacunae. The epithelium to alveolar crest distance is the distance from the inferior part of the epithelium to the alveolar crest.

Histology, slide scanning, digital imaging and Gram staining was performed at the Translational Pathology Core Laboratory (TPCL) at the David Geffen School of Medicine at UCLA. Gram staining was performed to identify bacteria. Bacterial quantification was measured in the ROI and normalized to the bone surface area. For osteoclast enumeration, tartrate-resistant acid phosphatase (TRAP) staining was performed utilizing the leukocyte acid phosphatase kit (387A-IKT Sigma, St. Louis, MO) and normalized to the bone surface area (66).

Statistics

Raw data were analyzed using GraphPad Prism (GraphPad Software, Inc. La Jolla, CA). Descriptive statistics were used to calculate the mean and the standard error of the mean (SEM). Data were analyzed by one and two-way ANOVA and post-hoc Tukey's test for multiple

comparisons, with statistical significance of 0.05. Socket healing was analyzed using the Fischer's exact test.

RESULTS

Experiment 1: Extraction of teeth with pulp exposure (PE)

First, we examined the effects of PE on MRONJ development. Most Veh (10/10) and ZA (9/10) treated animals displayed no clinical bone exposure (Fig. 1A, B). Radiographically, Veh animals showed healing with indistinct extraction socket borders (Fig. 1C-C1). ZA animals also showed osseous healing. However, the original extraction socket borders were easily discernible (Fig. 1D-D1). Furthermore, ZA treatment increased the Bone Volume over Tissue Volume (BV/TV) ratio in the area of the edentulous alveolar ridge (Fig. 1G). Histologic investigation of Veh animals showed normal epithelium, submucosa and healed sockets (Fig. 1E, F). ZA treated also animals displayed soft tissue healing. However, the extraction socket showed osteonecrosis, as indicated by confluent empty osteocytic lacunae. A statistically significant increase ($p<0.0001$) in percent osteonecrosis was observed (Fig. 1H).

Experiment 2: Effects of bacterial inoculation on periapical disease

Because extraction of teeth with PE did not lead to MRONJ lesions clinically, we inoculated the pulpal chambers with periapical pathogens creating experimental periapical disease (EPD), and examined its effects on the periodontium prior to tooth extraction. Radiographic examination of molars with PE revealed bone loss confined to the periapical region in Veh treated animals (Fig. 2A-A1). In Veh treated animals with EPD, large, widespread radiolucencies were visualized extending to near the inferior border of the mandible (Fig. 2B-B1). In ZA treated animals with PE, minimal alveolar bone loss was noted (Fig. 2C-C1). In ZA animals with EPD, the radiographic appearance was similar, with only slight widening of the apical periodontal ligament space (Fig. 2D-D1). Quantification of the root-alveolar bone distance demonstrated a

loss of apical bone in Veh animals with PE; this bone loss was significantly enhanced with EPD. The presence of EPD had no effect on the periapical bone loss in ZA treated animals (Fig. 2E). However, ZA treatment attenuated periapical bone loss. Histologic assessment paralleled radiographic findings. Veh treated animals with PE demonstrated periapical bone loss and inflammatory infiltrate around the apical area (Fig. 2F). In Veh animals with EPD, more extensive bone loss and prominent inflammatory infiltrate was noted (Fig. 2G). ZA treated animals with PE displayed minimal bone loss, with inflammatory infiltrate concentrated to the periapical region (Fig. 2H). While ZA treated animals with EPD displayed similar bone loss, a prominent inflammatory infiltrate extended beyond the periapical bone, into the surrounding alveolar bone (Fig. 2I, blue arrow).

Experiment 3: Extraction of teeth with EPD

We then tested soft tissue and osseous healing after extraction of healthy or EPD teeth. Visual examination revealed mucosal defects in 5% (1/20) of Veh animals with extraction of healthy teeth, 0% (0/16) of Veh animals with extraction of molars with EPD, and 12% (2/17) of ZA animals with extraction of healthy teeth (Fig. 3A, B, C, white arrows and Fig. 3E). In contrast, 62.5% (15/24) of ZA treated animals with extraction of teeth with EPD showed mucosal defects and clinical bone exposure (Fig. 3D, red arrow and Fig. 3E).

Radiographic assessment of the extraction sockets of Veh treated animals showed complete healing with remodeling of the socket outline (Fig. 4A-A1, B-B1, blue arrows). Most ZA treated animals with extraction of healthy teeth also showed socket with dense, woven bone, and a clear demarcation of the socket outline (Fig. 4C-C1, yellow arrows). In contrast, ZA treated animals with extraction of teeth with EPD presented partial or complete absence of osseous socket

healing (Fig. 4D-D1, red arrows) and periosteal bone formation along the lingual or buccal cortices (Fig. 4D1, white arrow). Qualitative assessment showed complete osseous socket healing in the majority of Veh treated animals, regardless of treatment, as well as ZA treated animals with extraction of healthy teeth (Fig. 4E). In contrast, 50% of the ZA treated animals with extraction of teeth with EPD showed absence of osseous healing, while 20% of animals demonstrated partial healing (Fig. 4E). Quantification of bone formation in the sockets showed statistically significant decrease in BV/TV in the ZA-EPD animals compared to all other groups (Fig. 4F).

Histologic assessment of extraction sockets from Veh treated animals with extraction of healthy or diseased teeth showed mucosal healing with normal keratinized epithelium and submucosa, and absence of inflammatory infiltrate. The extraction sockets were filled with woven bone with reversal lines (Fig. 5A, B, white arrows); osteonecrosis was not seen. ZA treated animals with extraction of healthy teeth also showed normal epithelial and submucosal lining over the extraction sockets (Fig. 5C). Sockets healed with woven bone and reversal lines, but limited remodeling of the socket outlines (Fig. 5C, white arrows), and areas of osteonecrosis were present (Fig. 5C1, blue arrows). In contrast, ZA treated animals with extraction of teeth with EPD demonstrated mucosal healing defects, with debris accumulation and epithelial migration extending to the underlying bone. Incomplete socket healing and remodeling, presence of osteonecrosis and sequestration of necrotic bone were also present (Fig. 5D-D1).

Quantification of histologic findings confirmed the qualitative assessment. ZA treated animals demonstrated a higher incidence of empty osteocytic lacunae and area of osteonecrosis compared to the Veh treated animals. Moreover, in ZA treated animals, extraction of teeth with EPD resulted in higher levels of empty osteocytic lacunae and osteonecrotic area (Fig. 5E, F). ZA

treated animals with extraction of teeth with EPD demonstrated decreased distance between the basal layer of the epithelium and the alveolar crest compared both to Veh treated animals, and ZA treated animals with extraction of healthy teeth (Fig. 5G). Finally, ZA treatment decreased the number of osteoclasts compared to the Veh treated animals (Fig. 5H).

Gram staining revealed general absence of bacteria within the submucosa or alveolar bone in Veh animals and ZA animals with extraction of healthy teeth (Fig. 6A-A1, B-B1, C-C1). In contrast, in ZA treated animals with extraction of teeth with EPD, bacteria were present around osteonecrotic areas and deeper within the alveolar bone (Fig. 6D-D2). Quantification demonstrated a significantly higher bacterial number in the ZA-EPD rats compared to all other groups (Fig. 6E).

DISCUSSION

Animal models, reported by us and others, have demonstrated an association between infectious dental disease and MRONJ (36,37,62,66,76,78). These models utilize experimental periapical/periodontal disease or spontaneously occurring peri-radicular disease combined with antiresorptives to characterize MRONJ lesions around affected teeth. Other investigators have followed a different approach to induce MRONJ lesions, by extracting healthy molars in animals treated with antiresorptives (71,79,80). Here, we combined the two, and utilized extraction of teeth with experimental periapical disease to observe the occurrence of MRONJ lesions in rats treated with high-dose ZA. We observed that extraction of teeth with EPD resulted in clinical, radiographic and histologic features of MRONJ; extraction of healthy or teeth with PE in ZA treated animals or in any Veh treated animals did not result in MRONJ lesions.

We made similar observations with spontaneous peri-radicular disease, reporting that only extraction of diseased teeth caused MRONJ lesions in mice treated with high-dose ZA (37). Our current studies in rats, however, offer several advantages, compared to our prior mouse study. First, EPD is amenable to experimental manipulation in relation to ZA treatment and disease duration; the existence and timing of spontaneous peri-radicular cannot be controlled. Furthermore, periapical disease is a well-established model that has been extensively used to investigate various facets of apical periodontitis, including microbial infection, immune responses, and host modulation of disease (81-83). In contrast, it is unclear if spontaneous peri-radicular disease, despite its pathologic radiographic and histologic features, parallels human disease. Finally, a larger animal model, with a more accessible oral cavity, makes intervention less technically challenging, allowing for more consistent and controlled disease development. In preliminary studies, extraction of teeth with drilled crowns in mice resulted in frequent tooth

fractures and retained root fragments due to the compromised crown integrity (unpublished data). Using rats instead of mice decreased the occurrence of such procedural difficulties. Furthermore, we excluded animals with retained root fragments from analysis, as they compromise socket healing.

Interestingly, we also reported defective socket healing after extraction of teeth with experimental periodontal disease in rats treated with ZA (38). While we observed poorly formed collagen fibers, increased levels of alpha-SMA, MMP-9, and MMP-13, all indicative of impaired healing, a great majority of animals presented without mucosal defects. Similarly, following extraction of teeth with pulp exposure in mice under antiresorptive treatment, no clinical bone exposure is noted despite the presence of osteonecrotic areas (36). Here, we made similar observations, after crown drilling and subsequent pulp exposure to the oral environment. However, we were only able to consistently induce clinical bone exposure after extraction of teeth with EPD, which were inoculated with species involved in periapical pathogenesis (84-86). These observations place great importance on the degree of inflammation in MRONJ pathogenesis. Indeed, μCT and histologic analysis confirmed this, reflected by an increase in bone loss and prominent inflammatory presence into the periodontal tissues in inoculated animals. Interestingly, ZA similarly inhibited periapical bone loss in the absence or presence of bacterial inoculation. However, when inoculated, inflammatory infiltrate within the marrow spaces of the periapical bone was noted. Subsequent extraction of inoculated teeth led to development of MRONJ lesions with soft tissue defects. In other studies, these mucosal defects are rare, and increase in prevalence with systemic therapy, such as immunosuppression (80). Similarly, the presence of MRONJ lesions following tooth extraction in ZA treated mice is

infrequent, and becomes apparent only when animals are treated with a chemotherapeutic agent (87).

Here, only animals with extraction of teeth with EPD displayed clinical bone exposure. Importantly, bacterial presence was seen around osteonecrotic areas of unhealed extraction sockets. Bacteria were noted not only on the exposed surface, but within the necrotic bone. Our observations parallel human findings that report the existence of bone infection (osteomyelitis) prior to tooth extraction in the majority of patients on nitrogen-containing bisphosphonates that subsequently developed MRONJ (80), as well as colonization of necrotic exposed bone by a variety of bacterial morphotypes (84,88-90). Indeed, others have also used bacteria to induce MRONJ lesions in mice. However, in these experiments, healthy teeth were extracted, and bacteria were added in the sockets after extraction (40). Our data, combined with such studies, demonstrate the significance of bacterial infection and associated inflammation in MRONJ pathogenesis.

Despite the resemblance to human disease, limitations of this model should be addressed. The young age of animals used in this study may affect wound healing following tooth extraction, as MRONJ largely presents in a more elderly population (91). Here, we utilized 200 µg/kg ZA twice weekly through the experiment, to induce MRONJ lesions. Indeed, this is a higher dose than clinically prescribed for malignancies; however, this increases disease prevalence, allowing for a smaller sample size to investigate disease development. Finally, rodents, although a powerful tool, have variances in bone remodeling, when compared to humans (92).

In summary, we present a model that captures the human MRONJ clinical scenario, and provides an experimental design that can be manipulated and modified in detail to allow the dissection of parameters involved in the process of MRONJ pathogenesis. The bacterial load and types, time

and dose of antiresorptives, time of experimental periapical disease, potential antibiotic treatment, endodontic treatment, and time of extraction are variables that can be easily adjusted to explore clinical outcomes. Supported by studies showing minimal MRONJ lesions in a healthy oral environment, we can begin to develop targeted therapies to prevent and treat this condition.

ACKNOWLEDGEMENTS

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FIGURES

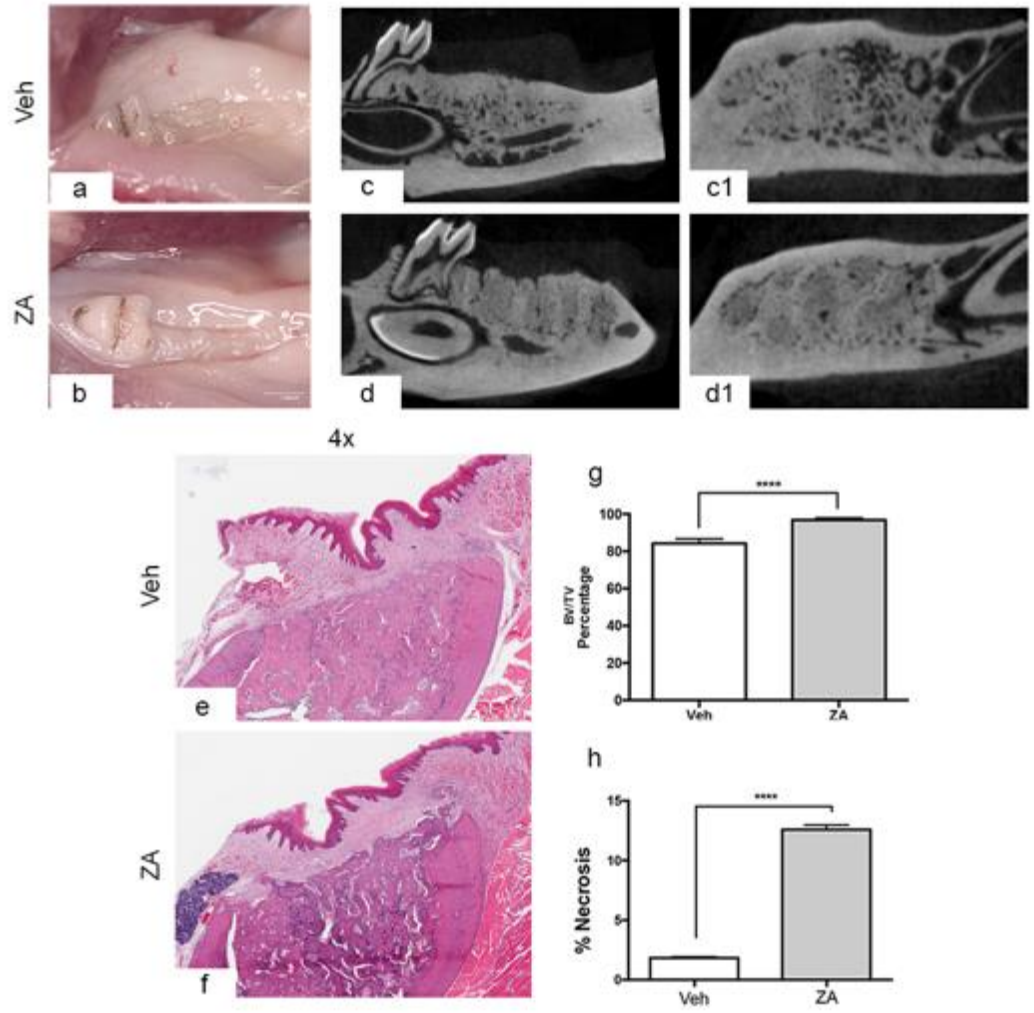

Fig. 1 Socket healing following extraction of teeth with pulpal exposure

Clinical images of socket healing following extraction of teeth with pulpal exposure in Veh (a) and ZA (b) treated animals. Saggital and axial µCT images of Veh (c, c1) and ZA (d, d1) treated animals with extraction of teeth with pulpal exposure. Representative H&E sections of extraction sockets in Veh (e) and ZA (f) treated animals with extraction of teeth with pulpal exposure. (g) Quantification of BV/TV percentage. (h) Quantification of percent osteonecrosis. Data represents mean value ± SEM. **** statistical significance, $p<0.0001$ (n=10 per group).

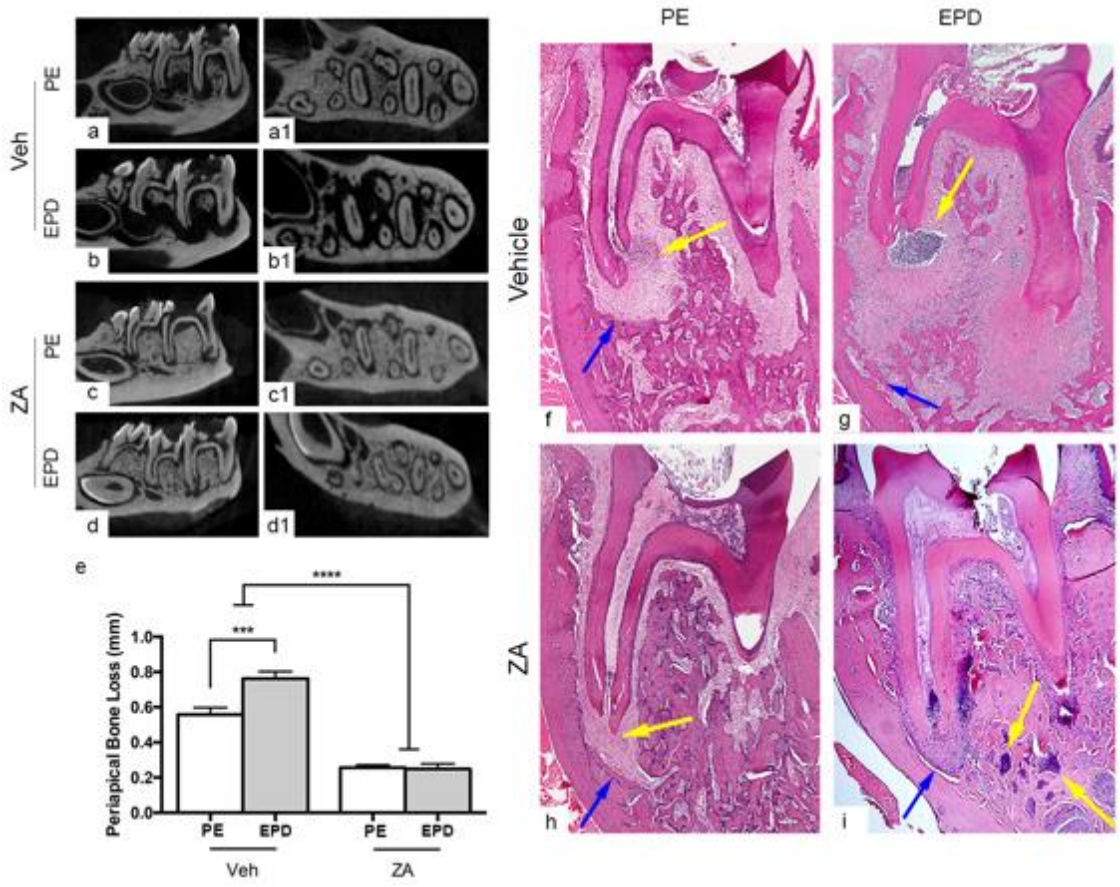

Fig. 2 Radiographic and histologic analysis of pulp exposure & EPD prior to extraction

µCT assessment of vehicle treated animals in the absence (a, a1) and presence (b, b1) of bacterial inoculation (EPD), and of ZA treated animals in the absence (c, c1) and presence (d, d1) of bacterial inoculation (EPD). (e) Quantification of periapical bone loss. Data represents mean value ± SEM. **** statistical significance, $p<0.0001$, *** statistical significance, $p<0.001$, (n=10 per group). Histologic assessment of periapical disease in the absence (f, h) and presence (g, i) of bacterial inoculation (EPD) in vehicle and ZA treated animals, respectively. Blue arrows point to the extent of periapical bone loss. Yellow arrows point to areas of inflammatory infiltrate.

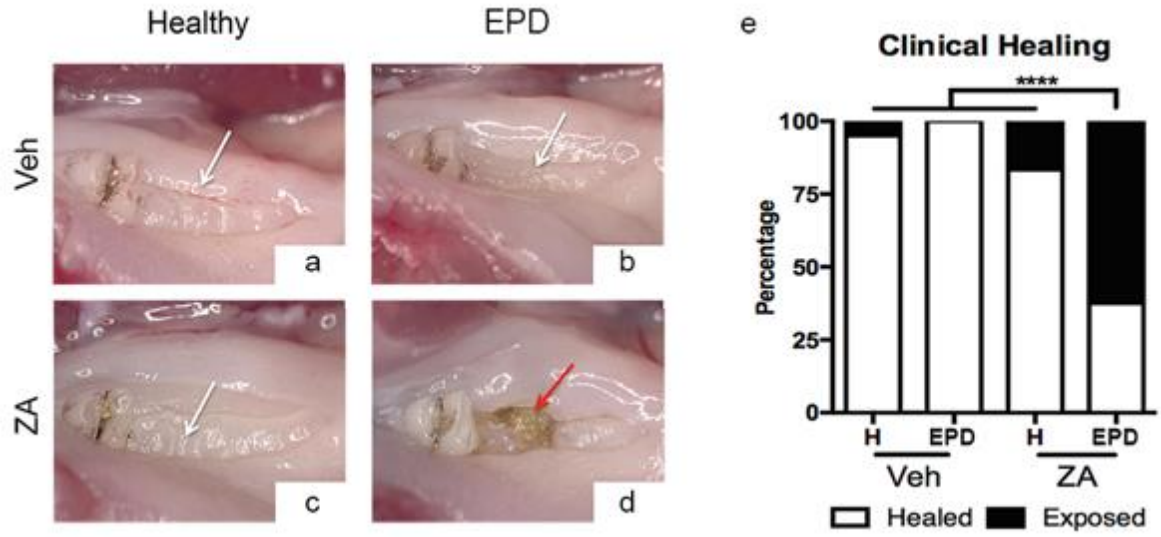

Fig. 3 Clinical Evaluation of Extraction Sockets

Clinical images of extraction socket healing in vehicle treated animals with the extraction of healthy teeth (a) or teeth with EPD (b), and ZA treated animals with the extraction of healthy teeth (c) and teeth with EPD (d). White arrows (a, b, c) point to healed extraction sockets. Red arrow points to an unhealed extraction socket with bone exposure (d). (e) Quantification of the percentage of animals with exposed bone or full healing. **** statistical significance, $p<0.0001$ (n=16-24 per group).

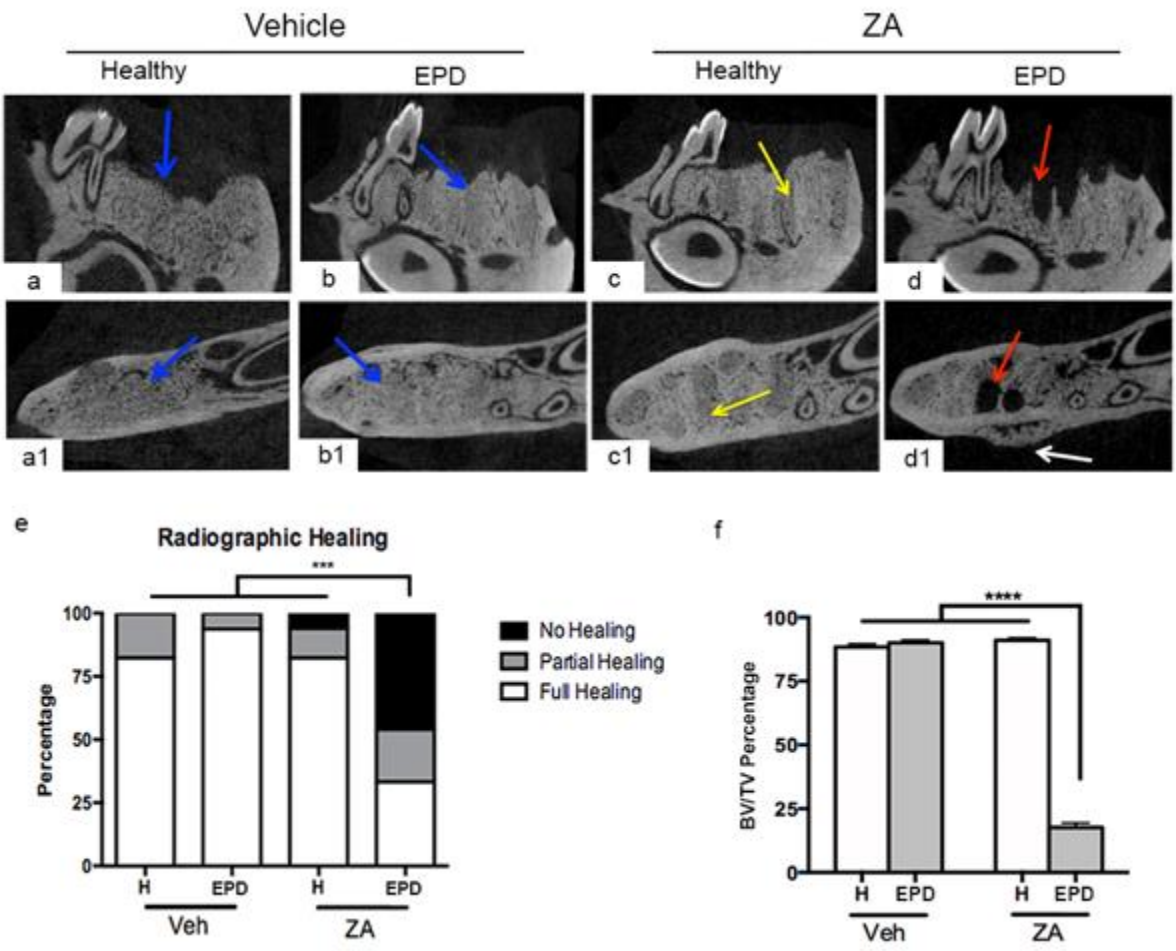

Fig. 4 Radiographic examination of extraction sockets

Sagittal and axial cross sections of µCT scans of vehicle treated animals with extraction of healthy teeth (a, a1) and EPD (b, b1). The blue arrows denote woven bone formation. Sagittal and axial cross sections of ZA treated animals with extraction of healthy teeth (c, c1) and EPD (d, d1). Yellow arrows point to areas of woven bone formation, demarcated from the outline of the extraction sockets. Red arrows point to empty extractions sockets. White arrow points to periosteal bone formation on the lingual cortex. (e) Quantification of radiographic healing. (f) Quantification of bone volume/tissue volume (BV/TV). Data represents mean value ± SEM. **** statistical significance, $p<0.0001$ (n=16-24 per group), *** statistical significance, $p<0.001$.

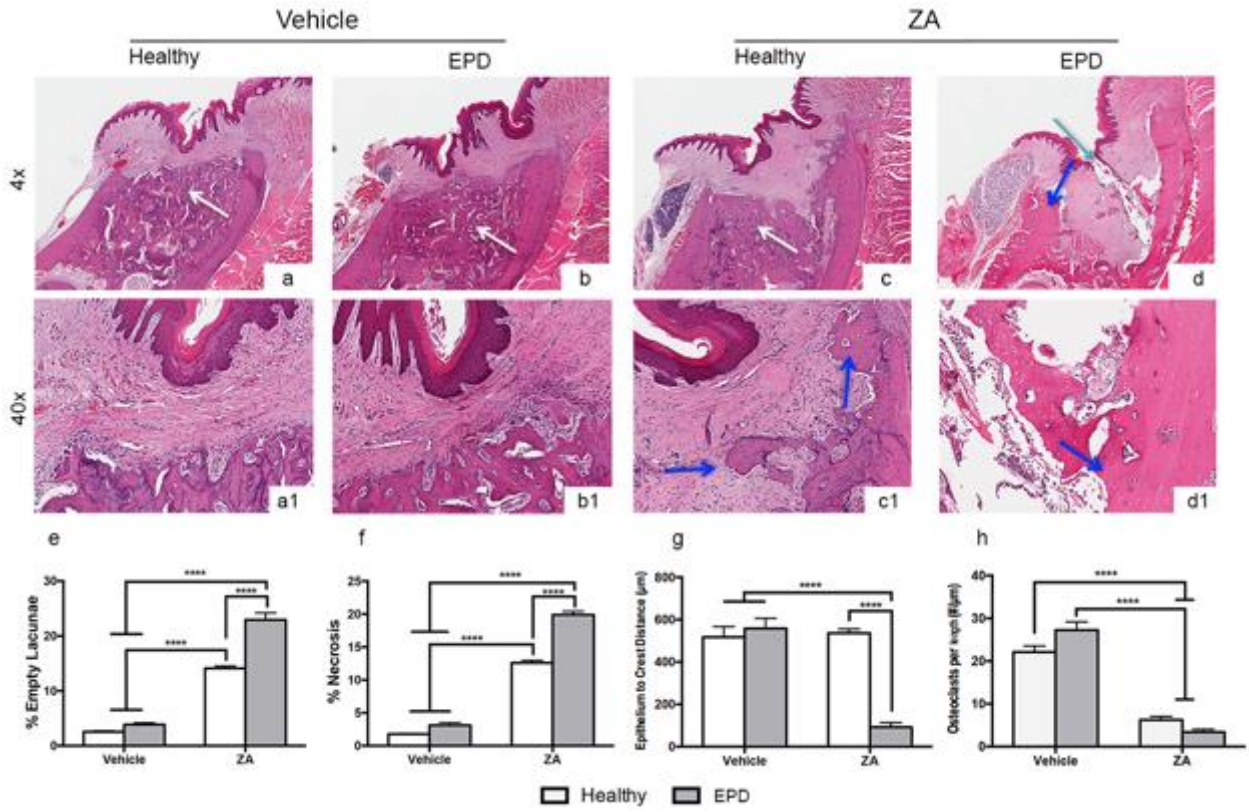

Fig. 5 Histologic evaluation of extraction sockets following extraction

Representative H&E sections of extraction sockets in vehicle treated animals with extraction of healthy teeth (a, a1) and teeth with EPD (b, b1). White arrows point to areas of woven bone formation. H&E sections of extraction sockets in ZA treated animals with extraction of healthy teeth (c, c1) and teeth with EPD (d, d1). Blue arrows point to areas of osteonecrosis. Cyan arrow points to mucosal defect, debris and bone exposure. Quantification of (e) percent empty osteocytic lacunae, (f) percent osteonecrosis, (g) epithelium to crest distance, and (h) osteoclasts/length. Data represents mean value ± SEM. **** statistical significance, $p<0.0001$ (n=15 per group).

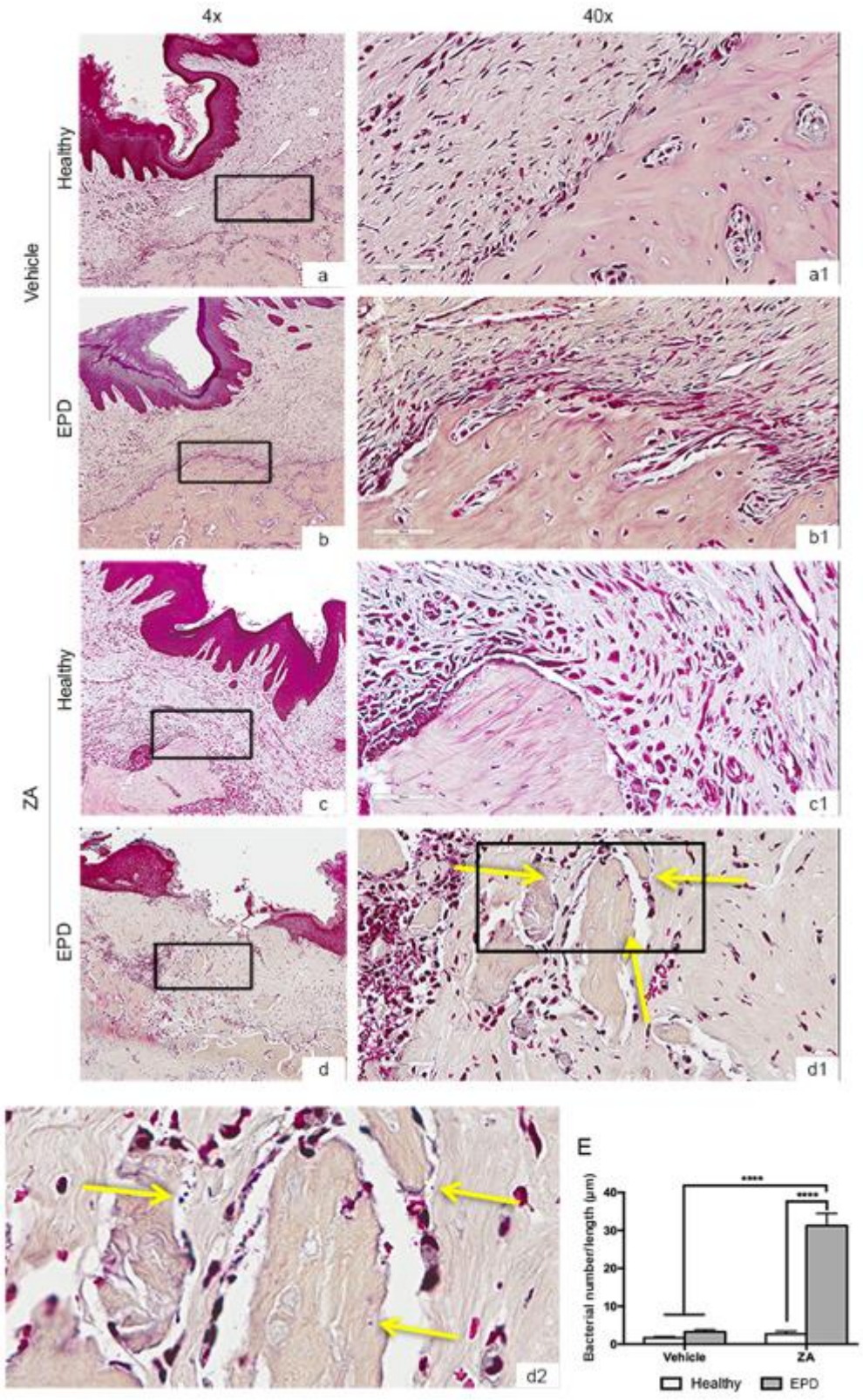
4x
40x
Vehicle
ZA
Healthy
EPD
Healthy
EPD
a
a1
b
b1
c
c1
d
d1
d2
E
Bacterial number/length (μm)
40
30
20
10
0
****
****
Vehicle
ZA
Healthy
EPD

Fig. 6 Gram staining of extraction sockets

Representative gram staining of extraction sockets in vehicle treated animals with extraction of healthy teeth (a, a1) and teeth with EPD (b, b1). Representative sections of extraction sockets in ZA treated animals with extraction of healthy (c, c1) and EPD teeth (d, d1). (d2) 100x magnification of the boxed area shows visible bacteria, denoted by the yellow arrows, seen around an osteonecrotic area. (e) Quantification of bacterial colonies per length. Data represents mean value ± SEM. **** statistical significance, $p<0.0001$ (n=8-10 per group).

Chapter 3: Clinically Relevant Doses of Sclerostin-Antibody do not Induce Osteonecrosis of the Jaw (ONJ) in Rats with Experimental Periodontitis

ABSTRACT

Antiresorptive agents, such as bisphosphonates and denosumab, are frequently used for the management of osteoporosis. Indeed, both medications decrease the risk of osteoporotic fractures; however, these medications are associated with rare, but potentially severe side effects, such as osteonecrosis of the jaw (ONJ). ONJ, defined as an area of exposed bone in the maxillofacial region that lasts for 8 weeks, often presents with significant pain and infection, and can lead to serious complications. Interestingly, other treatments for osteoporosis have been developed, such as antibodies against the osteocyte secreted protein, sclerostin. Sclerostin functions to inhibit the Wnt signaling cascade, leading to inhibition of bone formation. In clinical trials, a sclerostin-antibody (romosozumab, Amgen Inc., UCB Brussels) increases bone formation and lowers the risk of osteoporotic fractures. However, in conjunction with increased osteoblastic activity, a reduction in bone resorption markers is observed. This antiresorptive effect raises the concern of possible ONJ development in patients treated with sclerostin antibodies. Here, utilizing ligature-induced experimental periodontitis (EP), we evaluated the effects of sclerostin inhibition on the development of ONJ-like lesions in ovariectomized rats. Beginning eight weeks post-ovariectomy, rats were treated for 22 weeks with weekly injections of vehicle (Veh), 200µg/kg zoledronic acid (ZA), a potent bisphosphonate at 100-fold the osteoporosis dose, or 5mg/kg sclerostin antibody (Scl-Ab) at the osteoporotic dose. EP was initiated at Week 12 and maintained for the remainder of the study. Scl-Ab treatment transiently increased serum P1NP, a bone formation marker, in serum, increased BV/TV and decreased eroded surfaces in lumbar vertebrae. ZA treated rats developed histologic features of ONJ, while

Veh treated controls did not. Scl-Ab animals lost less periodontal bone in sites with EP. However, these animals presented with no histologic signs of ONJ. In conclusion, sclerostin inhibition enhanced structural bone parameters, without inducing ONJ-like lesions, in ovariectomized rats with EP.

INTRODUCTION

Osteoporosis, the most common metabolic bone disease, and osteopenia affect approximately 54% of US adults over 50 years of age, and are most common in postmenopausal women. Of this population, 20% are osteoporotic, requiring treatment, while the remaining 80% are osteopenic, for which treatment is neither currently approved, nor recommended (93). Prevalence increases with age, leading to osteoporotic fractures that are associated with significant morbidity and mortality, as well as decreased mobility (94-96). Management of osteoporosis largely focuses on antiresorptive agents, such as bisphosphonates (BPs), which inhibit osteoclast mediated bone resorption (97). Similarly, denosumab, a monoclonal antibody against receptor activator of nuclear factor kappa-beta ligand (RANKL), inhibits osteoclast development (98). Indeed, both types of medications reduce the risk of osteoporotic fractures (99,100). However, treatment with antiresorptives has been associated with infrequent, but significant side effects, such as osteonecrosis of the jaw (ONJ) and atypical femoral fractures (101).

ONJ is an area of exposed bone in the maxillofacial region that is present for at least 8 weeks, in patients taking antiresorptive or antiangiogenic medications (1,2). Although rare, ONJ can cause significant morbidity and present with pain, erythema and infection, often leading to a decrease in quality of life (1,102). Described in 2003, ONJ largely occurs in the presence of anti-resorptive or anti-angiogenic therapy, in conjunction with local risk factors, such as tooth extraction and dental disease. Despite this, the molecular level disturbances surrounding ONJ have not been well characterized (103). However, decreased bone resorption due to osteoclastic inhibition remains the central hypothesis in ONJ development (1,2).

Bone-forming agents for the treatment of osteoporosis also exist, and are analogues of parathyroid hormone (PTH), which function by directly stimulating bone formation through PTH

type 1 receptors (104-106). Indeed, in clinical trials, parathyroid hormone (1-34) decreased the risk of vertebral and non-vertebral fractures (105,107). Despite their anabolic functions, these drugs are only recommended for a total of 24 months due to an increased risk of osteosarcoma in rats (108). A more recent approach towards creating anabolic interventions for the treatment of osteoporosis has been to target sclerostin, an inhibitor of bone formation, through the use of monoclonal antibodies.

Sclerostin is a secreted glycoprotein of mature osteocytes and inhibits Wnt/beta-catenin signaling, attenuating osteoblast function and differentiation (109,110). In Phase I clinical trials, a sclerostin antibody (Scl-Ab, romosozumab, Amgen Inc., Thousand Oaks, CA & UCB Brussels, Belgium) not only showed increased bone formation markers, but also reduced levels of bone resorption markers (111). Similarly, in Phase II and III studies, romosozumab treatment increased bone mass, reduced vertebral, non-vertebral, and hip fractures, as well as decreased bone turnover markers over the 1 year treatment period (54,55). In another Phase III osteoporosis trial that reported reduced risk of osteoporotic fracture in the romosozumab group, two cases of ONJ were adjudicated in romosozumab treated individuals (53). With the occurrence of ONJ in romosozumab treated osteoporotic humans, the utilization of pre-clinical animal models, known to produce ONJ-like lesions, could be helpful in improving the understanding of this adverse event.

Here, we investigated the ability of an osteoporosis treatment dose of Scl-Ab to induce ONJ-like lesions using a well-defined animal model of experimental periodontitis (EP) in ovariectomized rats. We report that similar to vehicle (Veh) treated animals, and in contrast to animals treated with zoledronic acid (ZA), a potent BP at 100x its osteoporosis treatment dose, no ONJ-like lesions were observed in rats treated with the Scl-Ab in healthy sites or sites with EP.

MATERIALS AND METHODS

Animal care

This study was approved by the UCLA Chancellor's Animal Research Committee (ARC). Animals were housed following the ARC guidelines. Rats (1 per cage) were boarded in pathogen-free conditions with a 12-hour light/dark cycle. A standard diet (NIH-31 Modified Open Formula, ENVIGO, Madison, WI, USA) and water were provided *ad libitum*. A randomized, controlled, animal model design was utilized for this prospective study, following all the recommendations of the Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines.

Experimental design (Fig. 7A)

3-month old, female Sprague Dawley rats (n=120) were purchased from Charles River Laboratories and aged to 6 months old. All animals underwent ovariectomy (OVX) at 6 months of age (average body weight at OVX=384g). After 8-weeks of bone depletion, rats were randomly assigned (n=40 per group) by body weight to receive weekly intraperitoneal (IP) injections of vehicle (Veh), 200 μg/kg ZA (LKT Laboratories, St Paul, MN), or 5 mg/kg Sclerostin-antibody, consistent with dosage provided in clinical studies (55) and designed with the same complementarity-determining region as romosozumab, but with rat Fc construct to reduce immunogenicity (Scl-AbVI, Amgen Inc., Thousand Oaks, CA & UCB Brussels, Belgium). The average body weight at the first day of Veh, ZA, or Scl-Ab treatment was 468g. Treatment continued for 12 weeks; then, experimental periodontitis (EP), described below, was induced in the maxillae of 20/40 animals per treatment group (Veh, ZA, Scl-Ab). IP injections continued for 10 weeks, after which, animals were euthanized (Fig. 7A). The average weights at the induction of EP and necropsy were 520g and 528g, respectively. Each animal was treated as

an independent unit for analysis. All investigators were blinded during allocation, experimental protocols, animal handling, and measurements. Four animals (2 Veh-EP, 1 ZA-non-EP, 1 Scl-Ab-non-EP) died following OVX due to post-operative complications.

Serum analysis

1.0 mL of blood was collected via tail vein prior to OVX, the week prior to onset of medication delivery (8 weeks post-OVX), at 6 weeks after initiation of medication delivery (14 weeks post OVX), at 12 weeks after initiation of medication delivery (20 weeks post OVX) which was the time of induced EP, and at 22 weeks after initiation of medication delivery (30 weeks post OVX). Monitoring of serum levels of procollagen type I N-terminal propeptide (P1NP) was conducted in all animals using enzyme immunoassay (Rat/Mouse P1NP EIA, IDS; Fountain Hills, AZ, USA), as described (112) . No animals treated with Scl-Ab developed P1NP levels lower than the control average during the first 12 weeks of treatment, strongly suggesting that no animals developed antidrug antibodies.

Ovariectomy

All animals underwent bilateral OVX under sterile technique. A 2-centimeter incision was made at the midline of the dorsal surface, extending from the middle of the abdominal cavity to the superior portion of the hind legs. Blunt forceps were used to dissect through the subcutaneous fat, to the muscle layer surrounding the peritoneal cavity. The incision was moved to one side, and a 1-centimeter incision was made through the muscle, over the ovary. The ovary, ovarian fat pad, and uterine horn were located, then visualized outside the peritoneal cavity. A silk suture was used to ligate the uterine horn. Following ligation, the uterine horn was severed using electrocautery. The uterine horn, ovarian fat pad, and ovary were then removed. Hemostasis was confirmed, after which the remaining uterine tissue was returned to the abdominal cavity. A

resorbable 4-0 chromic suture was used to close the muscle layer. The skin incision was moved to the opposite side, and the same procedure was performed. Finally, a 4-0 non-resorbable suture was used to close the superficial incision. The superficial, non-resorbable sutures were removed under anesthesia 1 week after OVX, once wound healing was complete.

Experimental Periodontitis

EP was induced utilizing a well-established model of ligature-induced periodontitis (77,113,114). All rats were anesthetized using 2% isoflurane, delivered nasally. Then, a 4-0 silk suture (FST, Foster City, CA, USA) was ligated around the left maxillary second molar (M2) in 20/40 rats per group (randomly assigned based on body weight). All animals, including non-EP animals, were briefly anesthetized once weekly, to check and replace ligatures, as necessary. One ligature, in a Veh treated animal, needed to be replaced after 4 weeks of placement.

Ex-vivo µCT specimen scanning

Dissected maxillae, and vertebrae were imaged by micro-computed tomography (SkyScan 1172; Skyscan, Kontich, Belgium) using a 20-µm isometric voxel, as described (76,77). Bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), and trabecular separation (Tb.Sp) were measured using the third lumbar vertebrae. The region of interest (ROI) included the trabecular bone, 10 slices below the superior growth plate and 10 slices above the inferior growth plate of the third lumbar vertebrae. The terminology and measurements used are those recommended by the American Society for Bone and Mineral Research (ASBMR) (115). For scanned maxillae, volumetric data were converted to Digital Imaging and Communications in Medicine (DICOM) format and imported into Dolphin Imaging software (Chatsworth, CA, USA) for three-dimensional reconstructed images. The Cemento-Enamel Junction (CEJ) to Alveolar Bone Crest (ABC) distance was measured, as described (77). The CEJ-ABC distance

was measured at 4 sites, circumferentially, around the ligated 2nd molar in each animal. Bone loss was calculated by subtracting the average CEJ-ABC distance of healthy animals from the CEJ-ABC distance in each animal with EP. Since occurrence of spontaneous maxillofacial abscesses has been reported in rodents (66,116), any animals where food, hair, bedding, and debris impaction was observed clinically and coincided with radiographic changes in the absence of EP, or radiographic changes that extended to the 1st or 3rd molars beyond the ligature site, were excluded from the study; as a result, 1 maxillae in Veh-non-EP and 2 maxillae in Scl-Ab-non-EP treated animals were excluded from analysis.

20 animals per group (Veh, ZA, Scl-Ab: 10 non-EP, 10 EP) were used for vertebral endpoints, to verify sclerostin inhibition and ZA treatment. Radiographic and histologic endpoints for ONJ in the maxilla were made in 19 Veh, 19 ZA, and 17 Scl-Ab non-EP animals, and 18 Veh, 20 ZA, and 20 Scl-Ab EP animals.

Histologic processing

Maxillae were fixed for 72 hours in 10% phosphate buffered formalin (Fisher Scientific, Hampton, NH, USA), then transferred to 70% ethanol. Following µCT scanning, samples were decalcified in 14% ethylenediaminetetraacetic acid (EDTA) for 8 weeks. Samples were paraffin embedded and 5 µm coronal sections were made, then stained with H&E. The total number of osteocytic lacunae, empty osteocytic lacunae, total bone area, osteonecrotic area, and the distance from the ABC to epithelium were measured (77). Histology and imaging was performed at the Translational Pathology Core Laboratory (TPCL, David Geffen School of Medicine). Osteoclast enumeration was carried out using tartrate-resistant acid phosphatase (TRAP) staining (387A-IKT Sigma Aldrich, St. Louis, MO, USA). Positive cells were identified as multinucleated, TRAP-positive cells on the bone surface (77).

Bone histomorphometry

Animals were injected with 10mg/kg of 12mg/1mL calcein green (Sigma-Aldrich #C0875, St. Louis, MO, USA, C0875) in 2% sodium bicarbonate (pH 7.4) subcutaneously 10 and 3 days prior to euthanasia. Euthanasia was achieved using CO_2 inhalation followed by decapitation. OVX was verified during necropsy by confirming the absence of ovarian tissue and atrophy of uterine horn. All samples were fixed, as described above, after which the 5th and 6th lumbar vertebra (L5 & L6) were isolated, trimmed, dehydrated, and embedded in methylmethacrylate. Undecalcified parasagittal 4-μm-thick sections of the fifth lumbar vertebral body were prepared for histomorphometric measurements, as described (117). The ROI included the trabecular bone of the secondary spongiosa, 0.5 mm from the endosteal surface. Static and dynamic parameters, were assessed utilizing the Osteomeasure bone analysis software (Osteometrics, Inc.; Decatur, GA, USA). Eroded surface, defined as the crenated or lacunar bone surface, was assessed under polarized light to more clearly reveal the eroded lamellae.

Statistics

GraphPad Prism Software was used to analyze raw data (GraphPad Software, Inc., La Jolla, CA). P1NP serum levels before and after OVX were analyzed using a student's t-test, while time related changes were analyzed using a one-way ANOVA. Measurements were analyzed using a one-way ANOVA with post-hoc testing, with statistical significance set at $p<0.05$.

RESULTS

Serum analysis of P1NP

OVX treatment significantly decreased P1NP (Fig. 7B). No statistical differences were observed between P1NP in Veh and ZA treated animals; P1NP in ZA animals remained at Veh levels through the duration of the experiment. However, Scl-Ab animals demonstrated significantly higher levels of P1NP than Veh and ZA animals at week 6 and week 12 (Fig. 7C). P1NP in Veh animals significantly declined at 6 weeks and 22 weeks of treatment (Fig. 7C). ZA treatment significantly decreased P1NP, but only after 22 weeks of treatment relative to Week 0 (Fig. 7C). Scl-Ab treated animals, in contrast, demonstrated a significant increase in P1NP levels at 6 weeks of treatment that remained significant at 12 weeks (Fig. 7C). At 22 weeks of treatment, P1NP in Scl-Ab animals returned to the level of Veh and ZA animals (Fig. 7C).

MicroCT and histomorphometric analysis of lumbar vertebrae

µCT assessment showed an overall increase in trabecular bone mass of animals treated with ZA or Scl-Ab compared to Veh controls (Fig. 8, A-C).

ZA and Scl-Ab treatment significantly higher BV/TV (Fig. 8D) and trabecular number (Fig. 8F) compared to Veh control. For BV/TV, the Scl-Ab induced increase was statistically higher compared to the ZA effect. Scl-Ab treatment also caused a significant increase of the trabecular thickness compared to the Veh and ZA groups (Fig. 8E). Finally, trabecular separation was significantly higher in the Veh treated animals compared to the ZA or Scl-Ab treated groups. No difference between ZA and Scl-Ab treated groups was seen (Fig. 8G).

Bone histomorphometry revealed a significant decrease in mineralizing surface (MS/BS) with ZA treatment. In contrast, Scl-Ab treatment significantly increased the MS/BS compared to both Veh and ZA treated animals (Fig. 8H). Interestingly, a small but statistically significant increase

of the eroded surface (ES/BS) was noted in ZA treated animals. In contrast, in animals treated with Scl-Ab, a statistically significant decrease in ES/BS was present compared to both Veh and ZA treated groups (Fig. 8I).

Radiographic and histologic analysis of maxillae with EP

μCT analysis of healthy maxillary teeth in all groups revealed normal alveolar bone architecture, with no signs of ridge expansion (Fig. 9, A-A2, B-B2, C-C2). Animals with non-ligated molars, regardless of treatment, displayed a similar CEJ-ABC distance (Fig. 9G). Similarly, histologic examination revealed normal epithelial, submucosal and osseous architecture (data not shown). Analysis of animals with EP revealed differences in the alveolar bone levels around the ligated molar. Extensive bone loss circumferential of M2 to the mid-root level was noted in Veh treated animals. (Fig. 9D-D2, yellow arrows). Alveolar bone loss was not apparent in ZA treated animals with EP, as the CEJ-ABC distance remained similar to that seen in non-EP rats (Fig. 9G, H). In animals treated with the Scl-Ab, alveolar bone loss was attenuated, however, not completely abolished, as seen in ZA treated animals (Fig. 9F1, yellow arrows). Quantitative assessment of alveolar bone levels revealed a statistically significant increase in the CEJ-ABC distance in Veh animals with EP (Fig. 9G). However, a decrease in alveolar bone loss of the ZA and Scl-Ab treated animals vs. Veh animals was apparent (Fig. 9H). The difference in bone loss between ZA and the Scl-Ab treated animals did not achieve statistical significance.

Histologic analysis of Veh treated animals revealed alveolar bone loss at the buccal and palatal cortices around the area of the ligature (Fig. 10A). Epithelial rimming around the ligature was present. Inflammatory infiltrate was present in the submucosa between the basal aspect of the epithelial lining and the bone crest (Fig. 10A1, yellow arrows). Osteocytic lacunae containing osteocytes was evident in the alveolar bone (Fig. 10A1, bright green arrow). In contrast,

evaluation of ZA treated animals revealed a general lack of evidence of bone resorption. In addition, epithelial rimming, not only around the ligature, but also along the surface of the alveolar bone was noted (Fig. 10B, dark green arrow). Bone exposure was evident as regions of epithelial discontinuity over the underlying alveolar bone with the presence of debris (Fig. 10, B-B1, cyan arrow). Areas of necrotic bone, characterized by empty osteocytic lacunae were present in both the buccal and palatal cortices of the alveolar bone (Fig. 10B1, black arrows). Areas of dense inflammatory infiltrate were visible adjacent to the necrotic alveolar bone, around the area of the ligation (Fig. 10B1, yellow arrow). Scl-Ab animals demonstrated epithelial rimming of the ligature, a dense submucosa with inflammatory infiltrate, and mild alveolar bone loss (Fig. 10C, yellow arrows). The presence of osteocytes within the osteocytic lacunae of the alveolar bone was noted, with absence of osteonecrotic areas or bone exposure (Fig. 10C1, bright green arrow). Picrosirius red staining of Veh, ZA, and Scl-Ab treated animals identified alterations in collagen network organization around areas of EP. In Veh and Scl-Ab dense, well-ordered collagen fibers extended from the lamina propria into the alveolar bone (Fig. 10, A3-A4, C3-C4, purple arrows). In contrast, ZA treated animals demonstrated an absence of an organized collagen network, especially at the crestal part of the alveolar bone, where areas of osteonecrosis were noted (Fig. 10, B3, B4, white arrows).

When histologic sections of all animals were evaluated, epithelial discontinuity and bone exposure were absent in all animals with healthy periodontium, but were present in 0/18 Veh, 12/20 ZA and 0/20 Scl-Ab treated animals at the EP site ($p<0.0001$). Quantification of histologic findings revealed a significant increase of percent empty osteocytic lacunae and percent area of osteonecrosis in ZA treated animals compared to both Veh and Scl-Ab treated animals (Fig. 11A and 10B). No difference between Veh and Scl-Ab treated groups was present. The epithelium to

crest distance (Fig 9A1, black line), measuring the thickness of the submucosa overlying the periodontal bone was significantly decreased in ZA treated animals, in comparison to both Veh and Scl-Ab treated animals (Fig. 11C). Finally, quantification of osteoclast number showed a statistically significant decrease in ZA treated animals compared to both Veh and Scl-Ab groups (Fig. 11D).

DISCUSSION

ONJ was first described in 2003 and 2004 in patients treated with BPs (3,4); since then, it has also been reported in patients treated with denosumab, with the first such case occurring in 2010 (30). Although both BPs and denosumab inhibit bone resorption, they do so through distinct pharmacologic mechanisms. BPs inhibit protein farnesylation in mature, actively resorbing osteoclasts, leading to inhibited function and apoptosis, while denosumab binds to RANKL, inhibiting differentiation of osteoclast precursors, leading to decreased osteoclast formation, and thus, function (6,12). Despite their diverse mechanisms of action, ONJ prevalence and clinical presentation are similar in BPs and denosumab, highlighting the importance of osteoclastic inhibition in ONJ pathophysiology.

Though anti-resorptives are used as frontline therapy for osteoporosis, anabolic therapy is recommended for persons who have extremely low bone mineral density or continue to fracture on anti-resorptive treatment. PTH analogs, such as teriparatide, activate both osteoblasts and osteoclasts increase bone turnover, with an overall increase in bone mineral density and decrease in fracture incidence (104,106,118). Romosozumab, a monoclonal antibody against the Wnt inhibitor sclerostin, has been recently introduced as an alternative anabolic agent for the treatment of osteoporosis. Different than PTH analogs, in clinical trials, Scl-Ab demonstrates a dual effect on bone, increasing bone formation and decreasing bone resorption (55). While the anabolic effects of sclerostin inhibition are well documented and understood, the effects on osteoclastic function remain poorly explained, but data suggest this occurs downstream of Wnt signaling, a mechanism distinctly different from BPs and denosumab (119). Given Scl-Ab's osteoclastic inhibition, the possibility of adverse effects associated with its antiresorptive function cannot be excluded. Here, we used an animal model of ONJ to examine the effects of clinically-relevant

doses of Scl-Ab on alveolar bone. Veh treated animals served as the negative control, while animals treated with ZA at one hundred times greater than what is used clinically for osteoporosis, served as the positive control.

First, to confirm the effects of sclerostin inhibition, we investigated serum levels of P1NP, a marker used to assess bone formation. Serum levels in ZA and Veh animals declined through the duration of the experiment, with significance noted at 6 weeks and 22 weeks in Veh treated animals, and at 22 weeks in ZA treated animals. In Scl-Ab animals, a rapid increase in P1NP was noted, lasting for 3 months, but declined to levels just below week 0 at the end of the treatment period. This is consistent with the reported self-limiting nature of the increase in bone formation elicited by sclerostin inhibition, where bone formation peaks, and begins to fall 3 months following treatment initiation (120,121). While the mechanism of self-regulation of bone formation and bone mass is not yet well understood, multiple negative regulatory pathways appear to function downstream of canonical Wnt signaling to regulate bone formation (119).

Lumbar spine μCT measurements revealed increased bone structural values for both ZA and Scl-Ab treatments. These observations parallel results seen in OVX rats treated with Scl-Ab, as well as non-human primates (120,122,123), and parallels increases in bone mineral density seen in Phase III clinical trials of Romosozumab (55). To further confirm the anabolic effects of Scl-Ab treatment, we conducted histomorphometric assessment of the lumbar vertebrae. Indeed, a statistically significant increase in mineralized surface (MS/BS) was observed in the animals treated with Scl-Ab, when compared to Veh treated animals. Although P1NP levels had returned to Veh levels by 22 weeks, MS/BS indicated some sustained effects on bone formation were still present. This finding confirms published data showing that MS/BS is increased by Scl-Ab treatment in both pre-clinical and clinical studies (122). In comparison, ZA treatment significantly

decreased MS/BS, far below Veh levels, consistent with previously reported data in OVX rats and monkeys treated with BPs (124,125). Eroded surface in ZA treated animals was slightly, but significantly, higher than the Veh control group. This seemingly contradictory observation likely represents initiated resorptive sites early in treatment, when bone turnover would be highest in OVX rats. At this high dose of ZA, the resorptive site either has a protracted life span or is aborted, with failure of mature osteoclasts to effectively resorb BP containing mineralized bone (126). Together, these data agree with the well-established role of sclerostin inhibition in increased bone formation, and subsequent improvement in bone density.

The Scl-Ab induced bone formation was accompanied by a significant decrease in bone resorption, indicated by the attenuation of ES/BS values. These observations are consistent with published data, in various animal models. In cynomolgus monkeys, Scl-Ab treatment significantly reduces eroded surface (127). Similarly, in two independent models of OVX, Scl-Ab treatment decreases eroded trabecular surfaces through 26 weeks of treatment (112,120). Indeed, these findings parallel human data, where levels of the bone resorption markers, TRACP-5b and CTX, decrease (54,55). However, the mechanism through which sclerostin inhibition affects osteoclast function remains uncertain. In one study, treatment with a Scl-Ab led to increases in osteoprotegerin (OPG). Similarly, ex-vivo reports show reduced RANKL levels following sclerostin inhibition (119,121,128). In addition, Wnt signaling is known to regulate osteoclastogenesis (129). Finally, Scl-Ab treatment regulates expression of genes that control osteoclastogenesis in the osteocyte, such as the RANKL/OPG ratio, CSF, and WISP1 (119,130). In contrast, ZA animals had a statistically significant increase in eroded surface. Indeed, this is consistent with published studies, where BP treatment leads to an increase in eroded surfaces, likely due to osteoclastic activity prior to inhibition (126).

With these findings, we proceeded to investigate the local oral environment and potential development of ONJ-like features in the maxillae of Veh, ZA, and Scl-Ab treated animals employing a well-established model of EP. We and others have shown that EP in animals treated with high-dose antiresorptives induces ONJ-like lesions in rats and mice (63,77). Radiographic evaluation confirmed the effects of EP, seen as alveolar bone loss around the second molar of Veh treated animals. In ZA treated animals, we observed the inhibition of periodontal bone loss. Similarly, Scl-Ab treatment attenuated alveolar bone loss.

Other investigators have explored the effects of sclerostin inhibition on the regeneration of periodontal tissues after EP. Scl-Ab treatment improves indices of bone formation and enhances alveolar bone volume fraction in healthy rats or rats with ovariectomy induced osteoporosis (131,132). Interestingly, in the above studies, Scl-Ab treatment commenced after establishment of periodontal bone loss by ligature placement. Thus, the findings from these studies focus on the effects of sclerostin inhibition on the healing capacity of the periodontal tissues after removal of the instigating factor. In contrast, here, Scl-Ab was given while the ligatures were still in place, with ongoing periodontal inflammation. Collectively, these studies point to a potential role of sclerostin inhibition in the management of patients either with active periodontitis or during the regeneration phase after removal of the inflammatory factors.

Histologically, Veh treated animals showed bone resorption and inflammatory infiltrate in areas of EP. In contrast, in ZA treated animals, the alveolar bone was not resorbed and the presence of ONJ-like lesions, characterized by osteonecrotic areas and bone exposure, were noted. These findings are consistent with previous reports where dental disease and high dose antiresorptives induce ONJ-like lesions in rodents (36,62,63,77). Importantly, animals treated with Scl-Ab, demonstrated a comparable appearance to the Veh group with low levels of empty osteocytic

lacunae, a similar thickness of submucosa reflected by the epithelial to the alveolar crest distance, a well-ordered collagen network and absence of bone exposure.

The number of osteoclasts in the ZA treated animals were significantly attenuated. In contrast, Scl-Ab and Veh treated animals had similar osteoclast numbers. This was surprising given the reported effects of sclerostin inhibition in bone resorption and osteoclast function in patients or experimental animals with osteoporosis (54,55,111,115,121,122,133). However, in a setting of local inflammation in mice with rheumatoid arthritis, increased osteoclast numbers are observed in the joints of sclerostin deficient mice or mice treated with a neutralizing sclerostin antibody (134). Thus, it appears that the effects of sclerostin inhibition on osteoclast number and function is setting and possibly skeletal site dependent. For our experiments, the microenvironment of EP appears to overcome any potential systemic inhibition of osteoclastic function by sclerostin inhibition, in contrast to ZA. The distinct pattern of osteoclastic suppression around areas of EP that is associated with ONJ development was not observed in animals treated with Scl-Ab. As we and others have reported (63,66,76,77), areas of ONJ are only observed in ZA treated rodents around teeth with periapical or periodontal inflammation, but not around healthy teeth. The combination of osteoclastic inhibition with aggressive local inflammation appear to be key contributors to osteonecrosis, while bisphosphonates alone, without an instigating local factor, are not sufficient to induce such lesions.

While our study provides insight into the potential of a Scl-Ab to induce ONJ, we only tested the clinically relevant dose of Scl-Ab. As no supraphysiologic doses of Scl-Ab were tested, no definitive conclusions can be made about their potential to cause ONJ at high doses. While the ZA treated animals did develop ONJ, the dose used was one-hundred-fold above the dose used for osteoporosis, and was intended to serve as a positive control, to verify that our model can

indeed lead to the development of ONJ-like lesions in rodents. Because the incidence of ONJ in patients on osteoporotic doses of BPs is estimated at 0.001-0.01% (1), few animal studies investigating the ONJ incidence with osteoporotic BP doses in animals with experimental dental disease have been performed. In one such study utilizing spontaneous periodontal disease in rice rats (Oryzomys palustris) at an osteoporotic ZA dose, no gross ONJ-like lesions were observed; however, alveolar bone osteonecrosis was observed in 5% of the animals histologically (135). Furthermore, we treated our rats for 22 weeks. Although this is a relatively long treatment period compared to other ONJ studies, the possibility of ONJ development in experimental setting with longer treatment cannot be excluded.

In summary, our results supported an attenuation of the periodontal bone loss during active EP in osteoporotic rats administered the osteoporosis dose of Scl-Ab. These findings paralleled systemic bone-forming effects of sclerostin inhibition in the spine. Although sclerostin inhibition has been associated with attenuation of osteoclast function in the osteoporosis setting, this did not appear to be the case in the setting of EP, where local inflammatory signals overcome the inhibition of resorption mediated by activated Wnt signaling. Consequently, no radiographic or histologic signs of ONJ-like lesions were noted in sites with EP in animals treated with a Scl-Ab, similar to Veh animals, but in contrast to animals treated with ZA, at a dose that is one-hundred-fold above the ZA osteoporosis dose.

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AUTHORS' ROLES

Study design: DH, RWB, TLA, and ST. Study conduct: DH, IG, AS, OB, TLA, and ST. Data analysis: DH, IG, AS, RB, MS, DD, SD, FQP, TLA, and ST. Data interpretation: DH, IG, AS, RB, MS, DD, SD, FQP, TLA, and ST. Drafting manuscript: DH, TLA, and ST. Critically revising manuscript: IG, AS, OB, RB, MS, DD, SD, and FQP. Approving final version of manuscript: DH, IG, AS, OB, RB, MS, DD, SD, FQP, TLA, and ST. All authors had full access to data and take responsibility for the integrity of the data and accuracy of the data analysis.

FIGURES

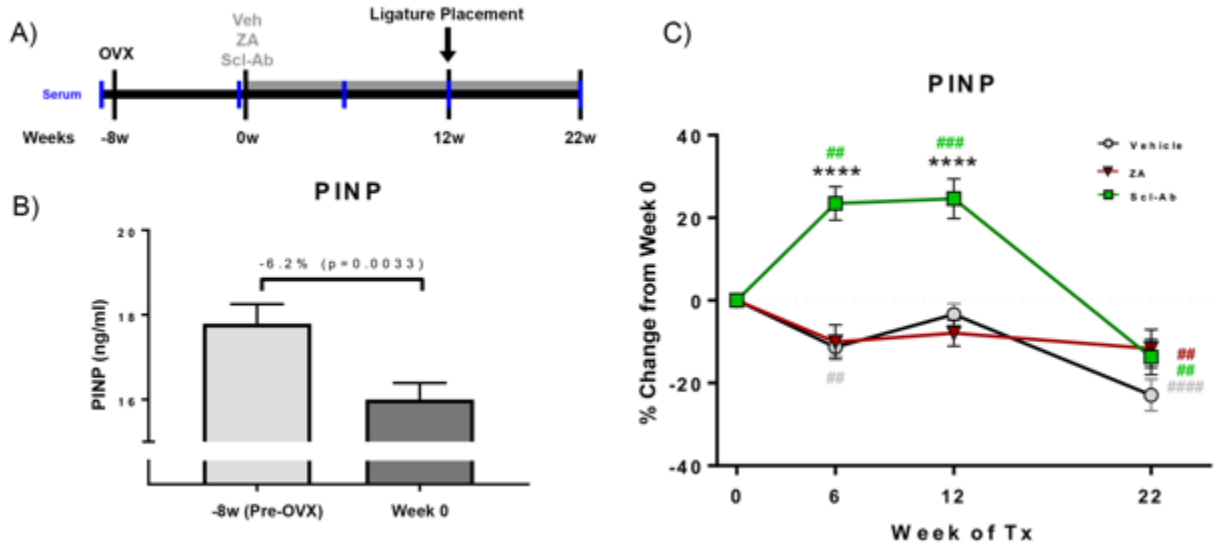
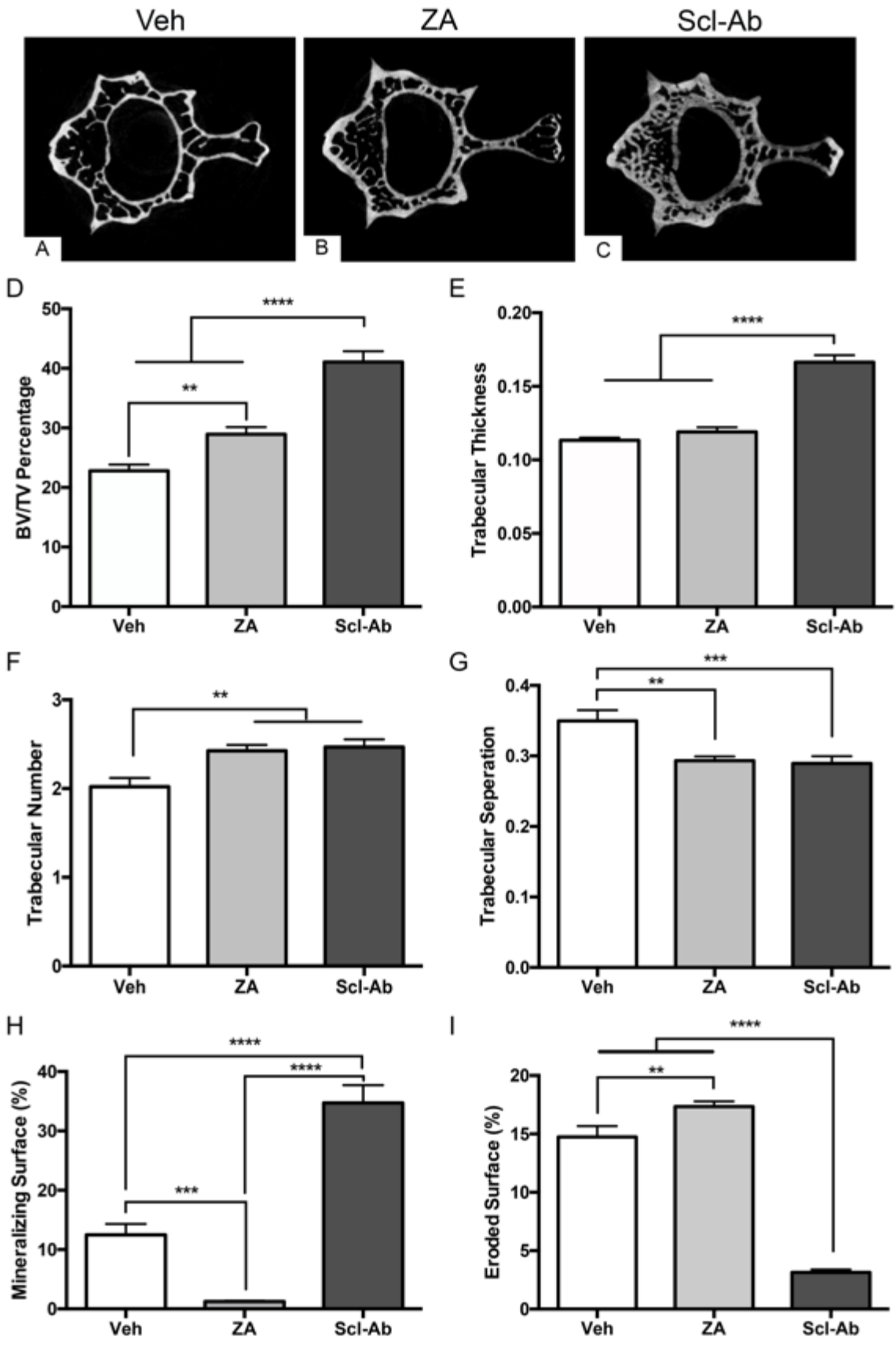


Figure 7: Experimental Timeline & Serum Analysis.

(A) Animals underwent OVX followed by an 8-week bone depletion period, after which Veh, ZA, or Scl-Ab treatment was immediately initiated. 12 weeks following treatment initiation, EP was induced. Treatment continued for 10 weeks after EP initiation, after which animals were euthanized. **(B)** Serum analysis of Pre-OVX (-8 week) and post-OVX (Week 0) P1NP. **(C)** Serum analysis of the bone formation marker, P1NP through the duration of the experiment. Data represents mean ± SEM: **** = *p<0.0001 when comparing Scl-Ab vs Veh or ZA animals. ## = p<0.01, ###= p<0.001, and #### = p<0.0001 when comparing each treatment group against Week 0 values.*


Veh
ZA
Scl-Ab
A
B
C
D
BV/TV Percentage
50
40
30
20
10
0
****
**
Veh
ZA
Scl-Ab
E
Trabecular Thickness
0.20
0.15
0.10
0.05
0.00
****
Veh
ZA
Scl-Ab
F
Trabecular Number
3
2
1
0
**
Veh
ZA
Scl-Ab
G
Trabecular Seperation
0.4
0.3
0.2
0.1
0.0
***
**
Veh
ZA
Scl-Ab
H
Mineralizing Surface (%)
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30
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I
Eroded Surface (%)
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15
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5
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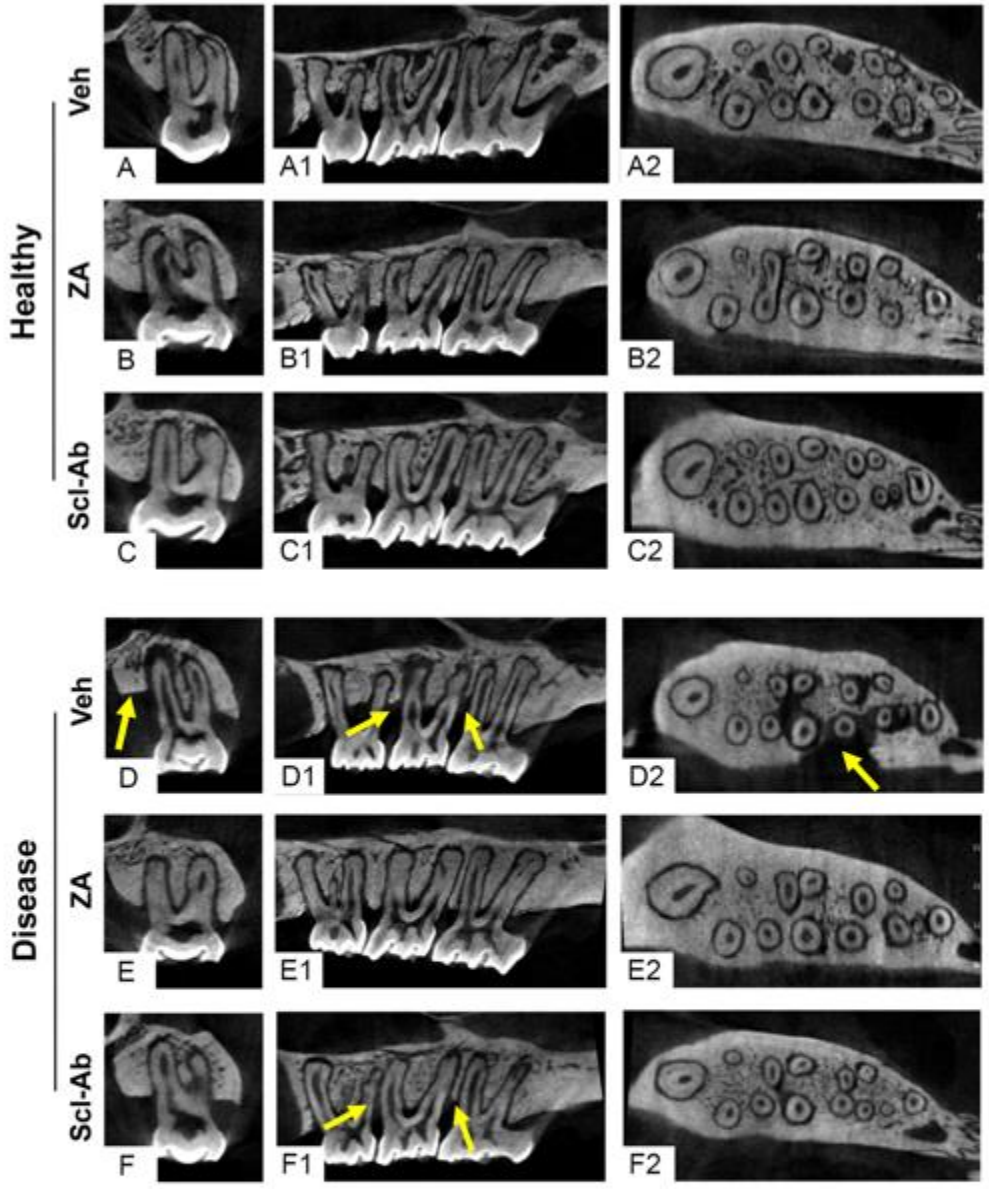
**
Veh
ZA
Scl-Ab

Figure 8: Radiographic Assessment of Lumbar Vertebrae.

Representative images of fourth lumbar vertebrae of **(A)** Veh, **(B)** ZA, and **(C)** Scl-Ab treated animals. **(D)** Quantification of bone volume fractionation (BV/TV). **(E)** Quantification of trabecular thickness (Tb.Th). **(F)** Quantification of trabecular number (Tb.N). **(G)** Quantification of trabecular separation (Tb.Sp). Histomorphometric quantification of **(H)** mineralized surface and **(I)** eroded surface. Data represents mean ± SEM: ****=$p<0.0001$, ***=$p<0.001$, **=$p<0.01$.


Healthy
Veh
ZA
Scl-Ab
A
A1
A2
B
B1
B2
C
C1
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Disease
Veh
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Scl-Ab
D
D1
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E
E1
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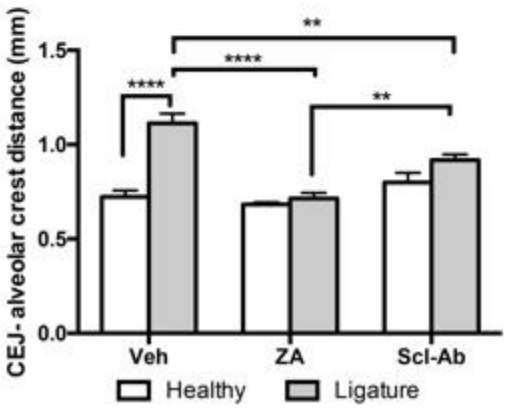
F
F1
F2

G


CEJ- alveolar crest distance (mm)
1.5
1.0
0.5
0.0
****
****
**
**
Veh
ZA
Scl-Ab
Healthy
Ligature

H

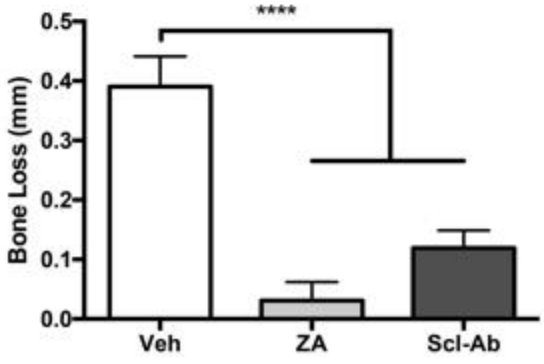

Bone Loss (mm)
0.5
0.4
0.3
0.2
0.1
0.0
****
Veh
ZA
Scl-Ab

Figure 9: Radiographic Evaluation of Maxillae with EP.

Representative coronal, saggital, and axial µCT images of healthy maxillae in **(A-A2)** Veh, **(B-B2)** ZA, and **(C-C2)** Scl-Ab treated animals. Representative coronal, saggital, and axial µCT images of maxillae with EP in **(D-D2)** Veh, **(E-E2)** ZA, and **(F-F2)** Scl-Ab treated animals. Yellow arrows point to arrows of alveolar bone loss. **(G)** Quantification of CEJ-ABC distance in healthy and EP animals. **(H)** Quantification of alveolar bone loss. Data represents mean ± SEM: ****=$p<0.0001$, **=$p<0.01$.

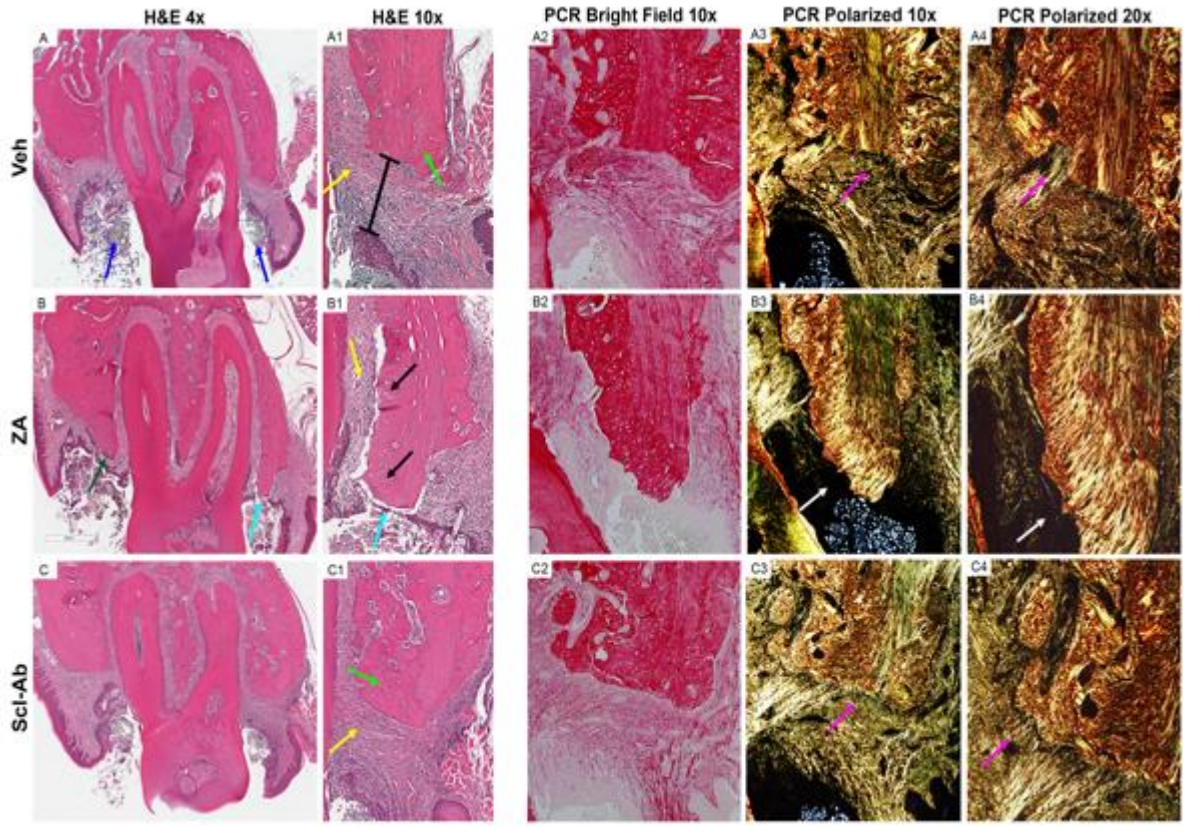


Figure 10: Histologic Analysis of Maxillae with EP

Representative coronal H&E images of maxillae with EP in **(A-A1)** Veh, **(B-B1)** ZA, and **(C-C1)** Scl-Ab treated animals. **(A)** Low power magnification of Veh treated animals with magnification of the buccal cortex shown in **(A1).** Low magnification bright field **(A2)** and polarized images **(A3)** of Picrosirius red (PCR) staining of the palatal cortex. High powered magnification at the junction of the alveolar bone and connective tissue **(A4)**. **(B)** Low power magnification of ZA treated animals with magnification buccal cortex shown in **(B1).** Bright field **(B2)** and polarized images of PCR staining of the palatal cortex **(B3, B4)**. **(C)** Low power magnification of Scl-Ab animals with magnification buccal cortex shown in **(C1)**. **(C2)** Bright field and **(C3, C4)** polarized images of PCR staining of the palatal cortex. Blue arrows point to the ligature. Yellow arrows point to areas of inflammatory infiltrate. Green arrows point to

osteocytes within osteocytic lacunae. The black bar represents the epithelial to alveolar bone crest distance. Purple arrows point to the attachment of collagen fibers to the alveolar bone. Cyan arrows point to area of bone exposure. Black arrows points to empty osteocytic lacunae. White arrows point to an area void of collagen, adjacent to an area of osteonecrosis.

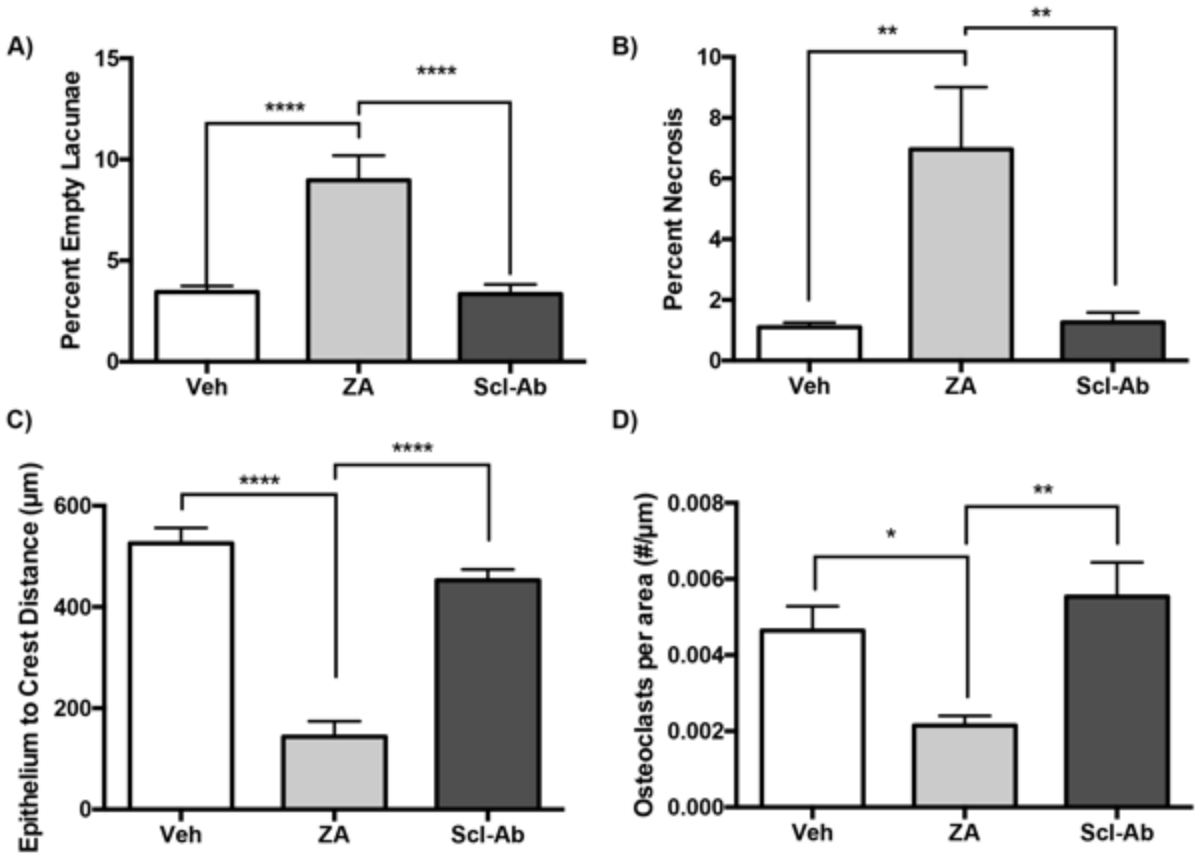


Figure 11: Quantification of Histologic Findings

Quantification of **(A)** percent empty osteocytic lacunae **(B)** percent osteonecrosis **(C)** epithelium to crest distance, and **(D)** osteoclasts per area. *Data represents mean ± SEM: ****=p<0.0001, ***=p<0.001, **=p<0.01, *=p<0.05.*

Chapter 4: Non-surgical Management of ONJ Utilizing Local Wound Care

ABSTRACT

Purpose: Medication-related osteonecrosis of the jaws (ONJ) is a known complication of antiresorptive medications with both surgical and non-surgical treatment options. The aim of this study was to evaluate the effectiveness of non-surgical therapy utilizing local wound care on management of ONJ lesions.

Methods: The authors conducted a retrospective-cohort study of patients that presented to the University of California, Los Angeles (UCLA) School of Dentistry, Oral and Maxillofacial Surgery Clinic for evaluation and treatment of ONJ. The primary predictor variable was wound care score; secondary predictors were demographics (age, gender), anatomic location, primary condition, type and time of antiresorptive treatment. Outcomes assessed were disease resolution and time to disease resolution. Statistical analysis was carried out Spearman's correlation for continuous/ordinal variables or Chi-square test for categorical variables. Time to event statistics, and Cox proportional-hazards models were calculated; finally, a Kaplan-Meier plot was generated to assess time to healing.

Results: 106 patients with 117 ONJ lesions were treated utilizing local wound; complete disease resolution was observed 71% of lesions, with an additional 22% of lesions undergoing disease improvement. Wound care score was significantly associated with disease resolution and time to resolution, while demographics, anatomic site, condition, type and time of antiresorptive treatment had no effect on resolution.

Conclusion: Local wound care increased the likelihood of ONJ resolution, and decreased the time to disease resolution. This strategy can be used in patients who cannot undergo surgery, and should be implemented in all patients with ONJ lesions who are managed non-surgically.

INTRODUCTION

Anti-resorptive agents, such as bisphosphonates and denosumab, are commonly used to treat osteoporosis and bone malignancies (136,137). Although the therapeutic effects have long been documented, side effects, specifically Medication Related Osteonecrosis of the Jaws (ONJ) can be serious and devastating (101). ONJ is defined as exposed bone in the oral and maxillofacial region for at least 8 weeks in the presence of anti-resorptives, without any history of radiation (1,2).

Osteoporosis and low bone density affect more than 50 million Americans older than 50, leading to at least 2 million osteoporotic related fractures (93,138). With the risk of ONJ in osteoporotic patients estimated at 0.001-0.15%, the use of anti-resorptives in these patients is warranted (1). Similarly, anti-resorptives have been very effective in decreasing skeletal related events in cancer patients (139,140). However, the incidence of ONJ increases to 1-16% in patients on oncologic doses of bisphosphonates or denosumab (1,141). While anti-resorptives are central in the management of osteoporosis and osseous malignancy, recent studies have shown that ONJ significantly decreases quality of life in many affected patients (60,102,139).

Once ONJ is established, current therapies vary, and are often dictated by the preference of the treating clinician, as shown by numerous treatments approaches in literature (1,48,50,142-144). The American Association of Oral and Maxillofacial Surgeons (AAOMS) treatment guidelines include antibacterial mouth rinse and pain control debridement for stage 0 and 1, while the more advanced stages 2 and 3 are treated by surgical debridement or resection (2). The American Society for Bone and Mineral Research (ASBMR) International Task Force on Osteonecrosis of the Jaws also recommends non-surgical therapy, reserving surgical therapy for more severe, refractory cases (1). Although these clinical guidelines exist, several studies advocate

debridement or surgical resection, regardless of disease stage with success rates ranging from 53-92% (49,50,58,143,145-147). However, more aggressive surgical approaches may not consider the morbidity associated with surgery in medically compromised patients. Studies of non-surgical treatment also exist, with disease resolution ranging from 18-90% (48,144). An overall challenge in comparing clinical approaches in the management of ONJ is the definition of disease resolution, which can vary from the absence of symptoms to complete mucosal epithelization.

Here, we present an alternative approach to traditional non-surgical therapy and surgical debridement or resection, focusing on local wound care of ONJ lesions. We defined disease resolution, as complete mucosal coverage, assessed clinically and lack of bony sequestrum, assessed radiographically. The purpose of this study was to investigate the effectiveness of non-surgical therapy, utilizing local wound care, on management of ONJ. The investigators hypothesize that non-surgical therapy, using local wound care, can lead to successful disease resolution in patients with ONJ lesions. The specific aim of this study was to measure the effectiveness of local wound care on disease resolution.

MATERIALS AND METHODS

Study Design/Sample

To address the research question, the investigators designed and implemented a retrospective-cohort study. The study population was composed of all patients presenting to a single clinician (TA) at the University of California, Los Angeles (UCLA) School of Dentistry Oral and Maxillofacial Surgery Clinic for evaluation and treatment of ONJ between June 2008 and July 2017. To be included in the study sample, patients had to be: 1) diagnosed with ONJ, confirmed clinically and radiographically, according to AAOMS guidelines and 2) return for follow-up appointments at the designated recall interval. Exclusion criteria were: 1) a failure to return for follow-up appointments (> 3 consecutive appointments missed), and 2) a pathologic fracture of the mandible at the initial consultation appointment.

Study Variables

The primary predictor variable measured in this study was local wound care score. ONJ lesions were assessed for the presence of bleeding at the affected site and plaque accumulation on the exposed bone. A scale of 1-3 was used for bleeding and plaque accumulation, with mild, moderate, and severe representing 1, 2, and 3, respectively. An overall wound care score from 1-6 was assigned by compiling the data measured (points for bleeding + points for plaque accumulation=total wound score). The wound care score used was obtained from the most recent clinic visit. If disease resolution occurred, the score from the visit prior to healing was used. Secondary variables assessed were demographics (age, gender), anatomic location, primary condition, type and time of antiresorptive treatment.

The primary outcome factors evaluated here was resolution of disease and time to disease resolution. Disease resolution was defined categorically, as disease progression, improvement, or

resolution. Disease progression was defined as an increase in disease stage, a larger area of infection or necrotic bone, an increase in the inflammation and/or infection of the exposed area, or pathologic fracture. Disease improvement was defined as lesser disease stage, or a decreased area of inflammation and/or infection. Disease resolution was defined as complete mucosal coverage with a lack of exposed, necrotic bone. Time to disease resolution was assessed, defined as a length of time, in months. In patients with disease resolution, this time was defined as the number of months needed for resolution of the disease. In patients with without resolution, this was defined as the number of months from the initial clinic visit until the last noted clinical visit.

Patient Management and Data Collection Methods

Patients were treated using the AAOMS guidelines, with modifications. No surgical intervention was performed other than removal of a detached, mobile sequestrum. Instead, local wound care, defined as mechanical, vigorous debridement and cleaning of exposed bone, was employed. All patients, at each appointment, regardless of staging or disease progression, were educated on the importance of oral hygiene, including local wound care, and routine dental care. Non-restorable teeth were referred for endodontic treatment, and the crown was then sectioned at the gingival level.

Patients were prescribed an antibacterial solution (chlorhexidine, 0.12%), but instructed to perform local wound care instead of oral lavage. Specifically, they were instructed to dip a cotton swab or small toothbrush in the chlorhexidine solution, and then vigorously mechanically clean, scrub, and debride the exposed bone or fistula to remove all visible plaque and debris. The clinician confirmed patient understanding following a brief demonstration. Patients were informed that discomfort, bleeding, and irritation, when cleaning, are expected, and will subside with continued treatment when inflammation and/or infection improves. This information was

also relayed to a primary caregiver or family members if the patient was physically limited or a lack of compliance was noted at subsequent appointments. Patients who received antibiotics were given a 3-week regimen of amoxicillin (500mg orally three times per day); if they were allergic to penicillin, doxycycline (100mg orally twice per day) was prescribed. A follow-up interval of 1, 2, or 3 months, was determined based on disease stage and symptomatology. Stage 1 patients with exposed bone, but no evidence of infection, were given chlorhexidine to be used for local wound care. Stage 2 and stage 3 patients with exposed bone, infection, pain, were given a course of antibiotics, analgesic medications, and chlorohexidine to be used for local wound care. Patients did not receive any other medications or growth factors.

Patient records were de-identified prior to data collection. The records were examined for demographics, underlying condition, type, duration, and dose of anti-resorptive use, clinical staging, and sites of disease. Anti-resorptive medications were only discontinued in three of the patients (with stage 1 ONJ for osteoporosis) per their primary doctor recommendation. All others remained on bisphosphonates or denosumab.

Data Analyses

Patient characteristics were compared between healing status (complete, improvement, progression) using Spearman's correlation for continuous/ordinal variables (e.g. age, stage) or the Chi-square test for categorical variables (Table 1, 2). Time to complete healing was compared using Cox proportional hazards (Cox PH) models and summarized against each effect with the estimated hazard ratio (95% CI). A full Cox PH model was completed using all terms (Table 3) to see if wound score was still significant after accounting for all other measured patient characteristics. A Kaplan-Meier plot was generated to assess the time to healing between patients

with high (≥ 3) vs low (< 3) wound scores (Figure 1). Statistical analyses were run using IBM SPSS V25, Armonk, NY. P-values <0.05 were considered statistically significant.

RESULTS

Patient population & demographics (Table 1)

106 patients with 117 ONJ sites were included in this retrospective study. The average patient age was 71.7 years. Age had no statistically significant effect on disease resolution. 71% of lesions were seen in females; sex had no effect on disease resolution. Similarly, ONJ prevalence was greater in the mandible. There was no effect on disease resolution based on anatomic location. 62% of lesions occurred in patients taking anti-resorptive medications for bone malignancies, while the remaining lesions were in osteoporotic patients. The underlying condition had no effect on disease resolution.

A majority of patients had been treated with a bisphosphonate, while only 15% had taken denosumab. No statistical difference in resolution was observed based on antiresorptive treatment. Interestingly, the duration of antiresorptive treatment had no effect on resolution status. While most ONJ lesions resulted from tooth extraction, all lesions healed equally, with no difference among inciting events. At initial presentation, the greatest number of lesions were stage 1. However, disease stage did not have an effect disease resolution. Of note, 1 patient with a stage 3 ONJ lesion in the mandible had a pathologic fracture after 2.5 years of local wound care; however, this patient had very poor compliance with home wound care (score=6) at all follow-up appointments.

Of importance, wound care score was the only variable associated with disease resolution. Patients with disease resolution had an average wound care score of 2.4, indicating mild levels of plaque and bleeding. In patients with refractory lesions, the average wound care score was 4.8, indicating high levels of bleeding and plaque accumulation. Interestingly, patients with disease improvement had an intermediate score of 3.7. Wound care score was highly associated with

disease resolution ($p<0.001$). Wound care was not associated with any other patient characteristic (Table 2). After adjusting for other patient demographics, wound care score was still highly significantly associated with disease resolution and time to resolution (Table 3). Finally, using Kaplan-Meir analysis, time to resolution was calculated. Patients with good wound care scores (< 3) healed at a much faster rate than those with poor wound care scores (≥ 3) than 3. At 24 months of treatment, only 20% of patients with good wound care (score <3) had unresolved lesions, while 60% of those with poor wound care scores (≥ 3) had unresolved lesions (Figure 12).

Clinical & Radiographic Manifestations of Representative Case

Patient with stage 2 ONJ

A 68-year-old male presented to the clinic with complaints of pain and purulent drainage for 8 months after extraction of tooth #31. On examination, exposed bone on the mucosa lingual to missing tooth #31 was noted with associated pain, erythema, and bleeding (**Fig. 13A**). Radiographically, areas of irregular cortication, diffuse trabecular sclerosis, and lack of remodeling of the extraction socket of tooth #31 were noted. However, no bony sequestrum was seen radiographically (**Fig. 13, D-D2**). Following 9 months of wound care, a large area of sequestrum exfoliated, revealing epithelized mucosa on the undersurface of the bone (**Fig. 13B**). The underlying tissue was fully epithelized at the 1 week follow-up visit (**Fig. 13C**). The follow-up radiograph displayed a well-defined defect in the socket of tooth #31, with no evidence of further sequestrum formation (**Fig. 13, E-E2**). However, sclerotic and lytic bone changes of the mandible were still apparent, with loss of bony support of tooth #30.

DISCUSSION

Here, we performed a retrospective analysis of local wound care, a novel non-surgical treatment, for the management of ONJ lesions. The purpose of this study was to evaluate if non-surgical therapy, utilizing local wound care, can be effective in the treatment of ONJ lesions. We hypothesized that local wound care can lead to disease resolution, without the need for surgery in patients with ONJ. Utilizing local wound care, 71% of ONJ lesions resolved completely with an additional 22% showing disease improvement. Specifically, the data show that this conservative management protocol is effective for ONJ treatment. Indeed, patients with better wound care scores had complete disease resolution at a faster rate than those with poor wound care.

Even though studies of ONJ treatment exist, none focus on local wound care for ONJ lesions, despite the well-established associations of poor oral hygiene and biofilm with ONJ development (144,148-150). Similarly, preventative dental measures prior to anti-resorptive treatment are of the utmost importance, and known to attenuate ONJ development (151,152). Following these observations, our treatment protocol centered on local wound care of exposed, necrotic bone. Current AAOMS treatment guidelines advocate the use chlorhexidine as an oral lavage (2). However, this lavage does not remove biofilm from the exposed bone, which acts as a continued source of irritation, inflammation, and often infection. For these reasons, patients in this study were instructed to use a cotton swab or small toothbrush dipped in chlorhexidine, and remove all plaque and debris from the exposed bone, which was defined as local wound care.

To assess the patient's wound care, we assigned a cumulative score measured by the presence of bleeding and plaque. Of importance, patients with disease resolution had a significantly lower wound care score, indicating low levels of plaque and bleeding. In contrast, patients who had refractory disease displayed significantly more bleeding and plaque. Furthermore, patients with

better wound care scores underwent disease resolution at a faster rate, with 60% of lesions completely healing within the first year. By 2 years of treatment, disease resolution had been obtained in 80% of patients with good wound care scores. In contrast, patients with poor wound care did not undergo disease resolution at a similar rate. At 1 year of treatment, only 20% of lesions had resolved; at 2 years of treatment, only 40% of lesions had resolved. This suggests that oral hygiene, specifically local wound care of ONJ lesions, is extremely important for disease resolution in patients with established ONJ.

In this study, demographics, anatomic site, underlying condition, disease stage, length and type of antiresorptive had no effect on disease resolution. initial disease stage had no significant effect on resolution. Interestingly, 94% (16/17) of lesions in patients on denosumab resolved. Although there are known differences between the pharmacologic effects of bisphosphonates and denosumab, no conclusions can be made due to the low sample size in these patients (6,12). Similarly, only 6 patients presented with stage 3 ONJ lesions; as such, any conclusions made for the treatment of these patients should be limited.

Importantly, disease resolution in our patients resulted from sequestration of the offending bone, with only 5% of resolved lesions recurring. In addition, wound care therapy duration was shorter for patients who initially presented with radiographic sequestra, as compared to patients without obvious radiographic signs. Local wound care eliminates the noxious, inflammatory environment surrounding exposed bone. In addition, follow-up CBCT scans demonstrated that osseous changes, including dense trabeculation, crater-like defects, and periosteal bone formation, remained underneath the healed mucosa. These findings suggest that although osteoclast inhibition was central in the initial formation of ONJ lesions, it was not required for subsequent mucosal healing after exfoliation of the sequestered necrotic bone. Since this abnormal osseous

architecture still remains under the healed mucosa, it would not be a suitable site for further surgical procedures, such as dental implants.

ONJ management is largely empirical, often focused on treating symptoms, rather than complete disease resolution. Treatment decisions are frequently based on general guidelines, without prospective clinical trials to support a single mode of therapy (2). An increasing number of studies advocate surgical management with disease resolution reported in 60-70% of cases (49,50,58,143,145-147). However, recurrence rates are widely varied, or unreported (50,153). In addition, the risk of intra and post-operative complications is increased during surgical treatment (154-157). It is important to note that metastatic cancer has been identified in ONJ specimens in 5.3% of patients taking antiresorptives for malignancies (51). Although rare, this must be considered in patients who undergo non-surgical therapy.

In contrast, studies of non-surgical management are few in number and have been less effective in disease management. This is further complicated by varied definitions of disease resolution, such as mucosal healing, lack of symptoms, or disease regression. Lerman et. al. reported 23% re-epithelization; another study defines disease resolution only as absence of pain, rather than mucosal healing, which may not be considered true resolution (144,158). A more recent study reported 65% resolution, but these patients discontinued anti-resorptive treatment, which may not be indicated for patients with malignancies or severe osteoporosis (159). A large multicenter study reports 25% disease resolution of 202 patients receiving non-surgical treatment for ONJ (160). Furthermore, a systematic review of 97 studies reports non-surgical, medical management of ONJ lesions leads to resolution in 45% of cases (161). Although no single treatment approach is proven superior, literature demonstrates that complete disease resolution occurs more often after surgical intervention (160).

Although we achieved complete disease resolution in 71% of patients, with an additional 22% undergoing disease improvement, there are indeed limitations to this study. ONJ is a rare event that occurs in a diverse patient population. Therefore, it is inevitable that this study included a heterogeneous population. However, the large number of patients allows evaluation of several variables, including the specific antiresorptive, disease stage, and primary diagnosis. Similarly, the resolution times may underrepresent total treatment time, as some patients were managed by other clinicians prior to presentation at UCLA. Furthermore, the retrospective nature of this study is subject to bias. Prospective studies with multiple treatment interventions (non-surgical vs. surgical) would be needed to establish the validity of this approach.

With the large sample size and single treatment modality, we have demonstrated that non-surgical therapy, utilizing local wound care, is a viable alternative to surgical intervention in patients with stage 1 and stage 2 disease, or patients who cannot tolerate a major surgical procedure. Indeed, prospective studies utilizing this approach will be conducted to confirm our findings here. Although ONJ is a serious disease with significant morbidity, here we have provided an alternative therapy to surgical treatment that provides disease resolution in the majority of patients. This can be easily implemented, and shifts the focus on patient care.

ACKNOWLEDGEMENTS

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TABLES

Table 1: Variables Associated with Disease Resolution & Time to Resolution

	Healing Status				**Time to Resolution**	
	Complete Resolution (number (%))	**Disease Improvement** (number (%))	**Disease Progression** (number (%))	**P-value**	**Cox Hazard Ratio (95% CI)**	**Cox p-value**
Age	72.0y	71.6y	70.4y	0.489	1.00 (0.98-1.02)	0.772
Gender				0.176	0.81 (0.49-1.65)	0.424
Female *Male*	63 (76%) 20 (59%)	15 (18%) 10 (29%)	5 (6%) 4 (12%)			
Anatomic Site				0.949	1.01 (0.64-1.59)	0.968
Maxilla *Mandible*	30 (70%) 53 (72%)	10 (23%) 15 (20%)	3 (7%) 6 (8%)			
Condition				0.487	1.20 (0.77-1.87)	0.417
Osteoporosis *Malignancy*	34 (75%) 49 (68%)	7 (16%) 18 (25%)	4 (9%) 5 (7%)			
Antiresorptive				0.079	0.80 (0.45-1.40)	0.434
Bisphosphonate	**65 (67%)**	**24 (24%)**	**9 (9%)**			
Alendronate *Zoledronate* *Other*	31 (72%) 31 (62%) 3 (60%)	10 (23%) 13 (26%) 1 (20%)	2 (5%) 6 (12%) 1 (20%)			
Denosumab	**16 (94%)**	**1 (6%)**	**0**			
Prolia *Xgeva*	10 (100%) 6 (86%)	0 1 (14%)	0 0			
Unknown	**2 (100%)**	**0**	**0**			
Staging				0.452	0.82 (0.58-1.18)	0.286
Stage 1 *Stage 2* *Stage 3*	47 (72%) 32 (70%) 4 (67%)	16 (25%) 9 (20%) 0	2 (3%) 5 (10%) 2 (33%)			
Time on Antiresorptive	5.5y	3.7y	4.8y	0.819	1.01 (0.96-1.06)	0.806
Inciting Event				0.269	-	0.686
Extraction *Spontaneous* *Dental disease* *Implant* *Denture*	50 (76%) 16 (68%) 7 (64%) 7 (64%) 3 (75%)	10 (15%) 9 (32%) 3 (27%) 2 (18%) 1 (25%)	6 (9%) 0 1 (9%) 2 (18%) 0			
Wound Care Score	**2.4**	**3.7**	**4.8**	**<0.001**	**0.32 (0.22-0.46)**	**<0.001**

Table 2: Variables Associated with Wound Care Score

Variable	Spearman	p-value
Age	-0.07	0.433
Gender	0.13	0.164
Anatomic Site	0.00	0.981
Condition	-0.10	0.268
Antiresorptive	0.10	0.291
Stage	-0.05	0.595

Table 3: Full Cox Proportional Hazards Model

Variable	Hazard Ratio (95% CI)	p-value
Age	0.99 (0.97-1.01)	0.433
Gender	1.06 (0.59-1.88)	0.583
Site	1.22 (0.75-2.01)	0.424
Condition	1.15 (0.61-2.17)	0.656
Antiresorptive	1.06 (0.59-1.91)	0.841
Stage	0.65 (0.41-1.01)	0.057
Wound Care Score	**0.29 (0.20-0.44)**	**<0.001**

FIGURES

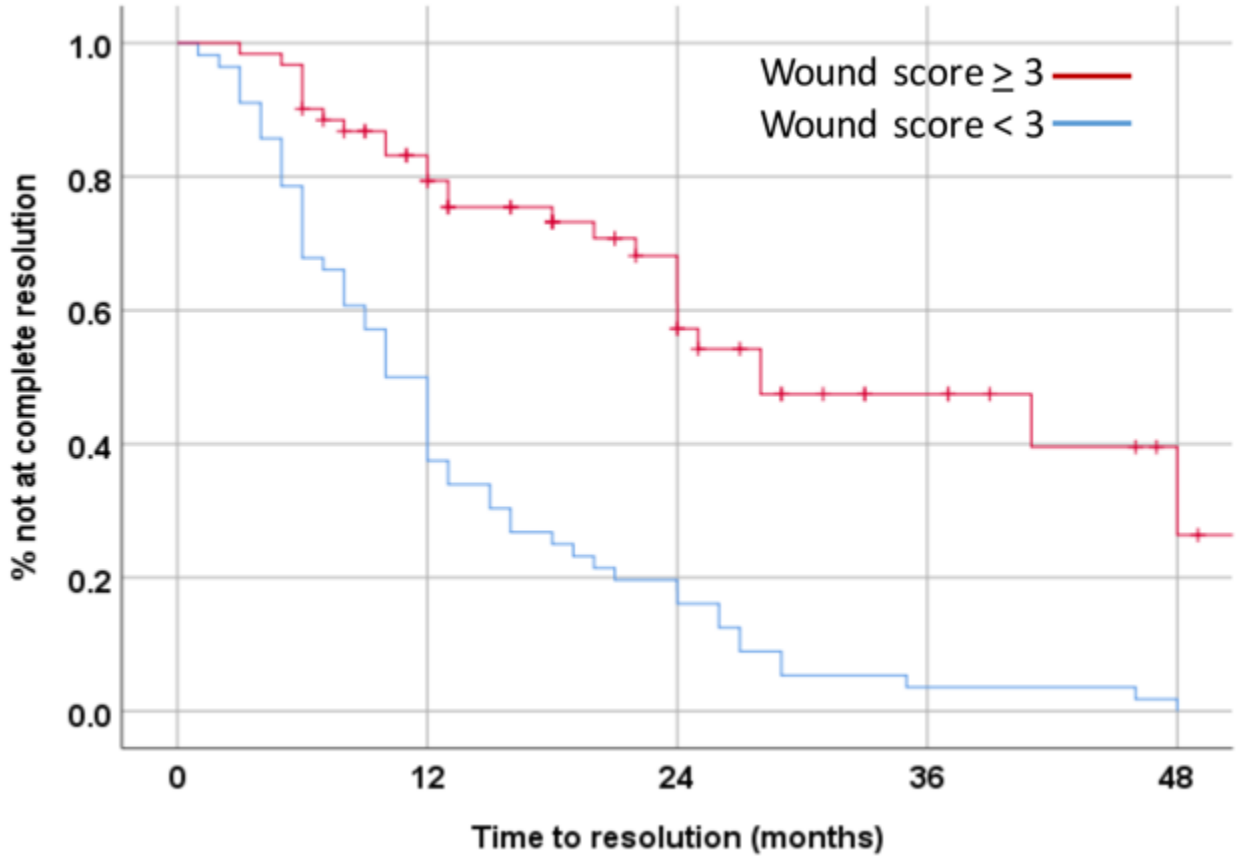

Figure 12. Kaplan-Meier plot of disease resolution status based on wound care score. Poor (≥ 3) and good (< 3) wound care scores were used to assess percentage of patients with unresolved lesions. Red line shows percentage of lesions without disease resolution as a function of time, in patients with poor wound care scores (≥ 3). Blue line represents percentage of lesions without disease resolution as a function of time, in patients with good wound care scores (< 3).

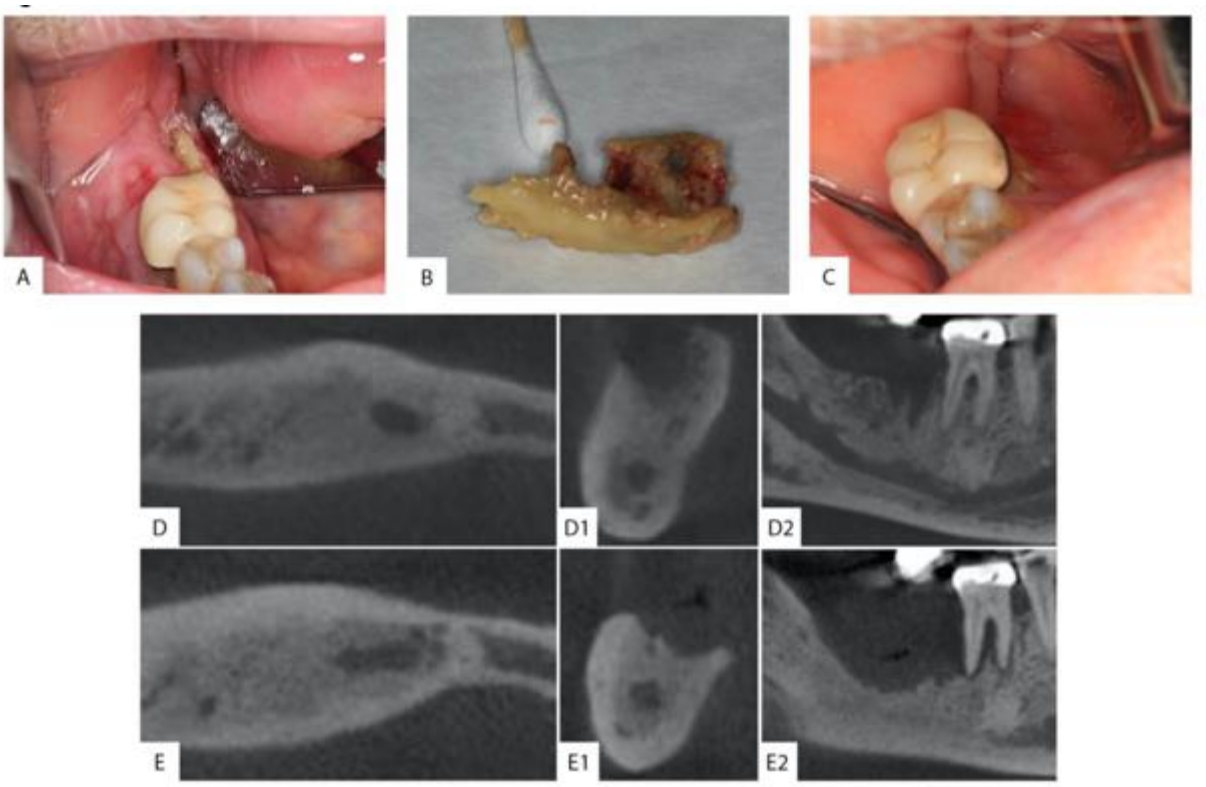

Figure 13. Clinical and radiographic presentation of a stage 2 ONJ patient. (A) Initial clinical presentation shows a small area of bone exposure on the lingual mucosa of the mandibular posterior teeth. (B) Exfoliation of necrotic bone following local wound care. (C) Clinical healing following exfoliation of necrotic bone. (D, D1, D2) CBCT scan at time of initial presentation demonstrates sclerotic changes in the area of the right posterior mandible, with an unhealed extraction socket. (E, E1, E2) Radiographic examination following sequestration shows a well-defined osseous defect, with increased sclerosis and lytic changes, as well as a loss of periodontal support around tooth #30.

Chapter 5: Osteoclast Related Osteonecrosis of the Jaw (ORONJ): Extending the Etiology of Osteonecrosis of the Jaw Beyond Medications

ABSTRACT

Osteonecrosis of the Jaws (ONJ), exposed bone in the oral cavity often presenting with pain or infection, was originally described as a side effect of bisphosphonate treatment. Since the original reports, other medications, including denosumab, and antiangiogenic or disease-modifying antirheumatic drugs (DMARDs) have been associated with the same rare clinical appearance. Thus, the designation Medication Related ONJ (ONJ) was introduced as a more inclusive diagnostic term. Although the term "ONJ" is based on the phenotypic presentation of non-healing exposed bone in the oral cavity, it potentially groups together conditions with distinct pathophysiologic mechanisms. Here, we present a cohort of three patients, seen at our institution, with history of exposed bone in the oral cavity that lasted more than eight weeks without history of radiation to the jaws. These patients satisfied the definitional criteria of ONJ, but had never been treated with any of the medications associated with ONJ. Instead, one patient suffered from osteopetrosis, and two patients suffered from pycnodysostosis, genetic diseases known to greatly affect the ability of osteoclasts to resorb bone. Detailed clinical, radiographic and histologic assessment showed striking resemblance of the oral exposed bone to that of patients with ONJ. Resorption pit assays confirmed attenuated bone resorption in these patients. These observations strongly support a central role of decreased osteoclastic bone resorption in ONJ pathophysiology. We introduce the term osteoclast related ONJ (ORONJ) to describe bone exposure in patients with either pharmacologic or genetic direct inhibition of osteoclast function. This terminology has an etiologic rather that symptomatology basis, complements the ONJ definition as it distinguishes between antiresorptive vs. other medications associated with ONJ,

predicts that new medications that would directly target osteoclastic function could induce ONJ, and allows the oral complications of patients with osteopetrosis and pycnodysostosis to be diagnosed as ONJ, and thus, guides their management.

INTRODUCTION

Osteonecrosis of the jaw (ONJ), osteomyelitis, osteoradionecrosis, alveolar osteitis (dry socket), or other less common conditions can lead to necrotic exposed bone in the oral cavity (162). ONJ is defined as exposed bone or bone that can be probed through an intraoral or extraoral fistula in the maxillofacial region that has persisted for at least eight weeks, in patients with current or previous treatment with antiresorptive or antiangiogenic agents and no history of radiation therapy or metastatic disease to the jaws (1).

ONJ is diagnosed clinically, based on the presence of exposed bone and pertinent medical history. Radiographic evaluation plays a confirmatory role and helps assess the extent of osseous changes, which often extend beyond what can be seen clinically. Radiographically, cortical erosion, lytic changes, trabecular bone sclerosis and non-healing sockets in the case of recent tooth extraction are consistent findings (163-165). Sequestrum formation and periosteal bone reaction can be present in more advanced cases (166,167). Histologically, necrotic bone with empty osteocytic lacunae, absence of bone remodeling, decreased number of reversal lines, lack of osteoblast rimming, bacterial colonization, and an inflammatory infiltrate have been described (168-170).

When the disease was first introduced in 2003 and 2004, all patients had been treated with various regimens of bisphosphonates (3,4) and thus, the term bisphosphonate related osteonecrosis of the jaws (BRONJ) was proposed (5,171). However, the 2014 position paper update by the American Association of Oral and Maxillofacial Surgeons (AAOMS), introduced a

nomenclature change to Medication Related ONJ (ONJ), due to an increase in the number of cases involving denosumab and antiangiogenic therapies (2).

This nomenclature change coincided with several publications that expanded the types of medications that are associated with bone exposure in the oral cavity. These medications range from antiresorptives and antiangiogenics to chemotherapeutics and disease-modifying antirheumatic drugs (DMARDs) (9-11,172). As evidence accumulates, more medications appear to be associated with the presence of exposed bone in the oral cavity that falls under the definition of ONJ. While "ONJ" is a useful and practical clinical term, it does not distinguish the underlying pathophysiology, which risks grouping together conditions that could require different treatment strategies or have different prognoses.

Despite being described nearly 15 years ago, the pathophysiology of ONJ remains largely unknown. Osteoclastic inhibition, suppressed bone turnover, compromised mucosal immunity, infection with specific bacteria, inhibition of angiogenesis, interruption of vascular supply, or direct soft tissue toxicity have been proposed. It is likely that more than one of these processes are involved in ONJ pathogenesis, particularly in patients treated with diverse classes of pharmacologic agents.

Here, we present three cases of patients with bone exposure in the oral cavity with clinical, radiographic and histologic features indistinguishable to those of ONJ patients. However, none of these patients had received any antiresorptive, antiangiogenic or DMARD medications, none had received radiation to the jaws and none had metastatic cancer. Rather, these three patients suffered from osteopetrosis or pycnodysostosis, both of which are genetic diseases that disrupt

osteoclast function (173). These clinical observations provide strong evidence for the central role of osteoclasts in ONJ pathophysiology. Thus, we propose a new classification scheme that defines Osteoclast Related ONJ (ORONJ) as a distinct subgroup of ONJ patients.

MATERIALS AND METHODS

Patient Sample & Treatment

Patients were referred to the UCLA School of Dentistry Oral and Maxillofacial Surgery (OMS) Clinic for evaluation and treatment of non-healing areas of exposed bone. Three patients were included in this study: one patient diagnosed with osteopetrosis and two patients diagnosed with pycnodysostosis. A total of four age and gender matched controls were used for in-vitro studies. This study was approved by the UCLA Institutional Review Board (IRB).

The diagnosis of ONJ was made following the AAOMS and ASBMR guidelines (1,2). Panoramic radiographs were obtained using the Veraviewepocs 2D (J Morita, Irvine, CA, USA) and Cone Beam Computed Tomography (CBCT) scans were obtained using the 3D Accuitomo 170 scanner (J Morita, Irvine, CA, USA).

Isolation of Peripheral Blood Mononuclear Cells and Resorption Pit Assays

Isolation of peripheral blood mononuclear cells (PBMCs) and resorption pit assays were performed using standard methodologies (174,175). Briefly, fresh blood was collected into an anticoagulant vessel, and centrifuged at 400g for 40 min in a Ficoll-Paque gradient (Sigma Aldrich, St. Louis, MO, USA). The mononuclear cell layer was isolated, diluted in PBS, respun and re-suspended in culture media.

$2x10_6$ cells were plated in alpha-MEM with 10% FBS and 1% antibiotics (100 U/ml penicillin and 100µg/ml streptomycin) on 24-well OsteoAssay plates (Sigma Aldrich, St. Louis, MO, USA). Plates were supplemented with 25ng/ml recombinant human Macrophage Colony-

Stimulating Factor (M-CSF) (PeproTech, Rocky Hill, NJ, USA) and 50ng/ml Receptor Activator of Nuclear-factor Kappa-beta Ligand (RANKL) (PeproTech, Rocky Hill, NJ, USA). Media was replaced twice per week. After 14 days of culture, plates were stripped of cells and Von Kossa staining was performed. Plates were imaged at 2x magnification. Total resorption pit area was visualized under light microscopy and measured using cellSens imaging software (Olympus, Center Valley, PA).

Statistics

GraphPad Prism Software was used to analyze raw data (GraphPad Software, Inc., La Jolla, CA). Data was analyzed using a student's t-test, with statistical significance set at $p<0.05$.

RESULTS

Clinical Presentations

A 34-year-old male with osteopetrosis (Patient 1) was referred to the OMS clinic by his general dentist for evaluation of an area of non-healing bone in the upper right quadrant of several months duration. The patient denied any medical conditions, other than osteopetrosis, and did not take any medications. The patient measured 5'10" and weighed 183 lbs; he was unable to ambulate without the utilization of crutches. He reported having three teeth in the maxillary right quadrant (the maxillary second premolar and the maxillary first and second molars) removed 2 years ago. He reported continuous irritation, pain, and bleeding around this non-healing area since. Upon examination, a large area of exposed bone was observed in the maxillary right quadrant (Fig. 14A).

A 45-year-old female with medical history significant for pycnodysostosis (Patient 2) presented for evaluation of exposed bone associated with constant pain and irritation. The patient denied taking any medications. The patient was 4'2" and weighed 95 lbs. The patient reported having all of her teeth removed four years ago, and complained of an area of non-healing bone at the area of the extraction sites. After six months of follow-up with her general dentist with no resolution of the lesion, the patient was referred to the OMS clinic for evaluation. Examination revealed an area of exposed bone with surrounding erythema on the lower right quadrant (Fig. 14B). Additionally, a high vaulted palate with an oro-nasal fistula were present.

A 49-year-old female with pycnodysostosis (Patient 3) presented for evaluation of an area of exposed bone associated with constant pain and irritation. The patient was 4'1" and weighed 101

lbs. She reported no other significant medical history, and was not taking any medications. Her dental history was mostly uneventful. However, she recently reported that a piece of her tooth broke, after which she noted an irritation in the right maxillary quadrant. Upon examination, an area of exposed bone on the buccal surface of the maxillary right premolars was observed (Fig. 14C). The patient has been followed for over two years, with no resolution of the bone exposure.

Radiographic Examination

Radiographic assessment revealed increased bone density and trabecular bone sclerosis in all patients (Fig. 15, white arrows). Cortical erosion was visible on the buccal and lingual surfaces of all patients (Fig. 15, yellow arrows). Non-healing sockets were visible in the patient with osteopetrosis with history of tooth extraction in maxillary right quadrant (Fig. 15, cyan arrows). For the same patient, the radiographic changes extended to the floor of the maxillary sinus, which appeared discontinuous and irregular (Fig. 15, orange arrows). Sequestra formation was visible around most areas of exposed bone, sometimes encompassing the entirety of the tooth (Fig. 15, red arrows). Lytic changes were apparent in all patients (Fig. 15, red arrows).

Additionally, multiple impacted teeth were present in patients 1 and 3. Patients 2 and 3 presented prominently obtuse mandibular angles, a characteristic appearance of patients with pycnodysostosis (176).

Histologic Examination (Fig. 16)

Histologic examination of all patients showed multiple fragments of lamellar bone with widespread empty osteocyte lacunae, indicative of osteonecrosis. No histologic features of

remodeling were present; specifically, no reversal lines, no osteoblastic rimming, no osteoclasts or erosive surfaces were noted. Some of the samples showed bacterial colonies and a minimal to mild associated acute (neutrophilic) and chronic (lymphoplasmacytic) inflammatory response.

Resorption Pit Assays

Less than 5% of the plate was resorbed when PBMCs from Patient 1 were used. Pinpoint areas of resorption were visible, but no osteoclast tracks were present. In comparison, age-gender matched controls resorbed 44% of the plate, with extensive resorptive pits and associated osteoclast tracks were noted (Fig. 17 A-C, H). A reduced resorptive capacity was also noted when PBMCs from Patients 2 and 3 were analyzed. Areas of resorption were negligible, and when apparent, lacked osteoclast tracks. In contrast, cells from age-gender matched controls demonstrated significant resorptive activity (Fig. 17 D-G, H). The negative control wells, not supplemented with RANKL, showed no resorptive capacity in any patients or control samples (data not shown).

DISCUSSION

Osteoclasts play a central role in bone homeostasis, and coordinate with osteoblasts and osteocytes to maintain bone structure and function (177,178). Unbalanced osteoclastic activity compromises osseous structure and is frequently present in conditions that affect the skeleton (179). In such disorders, inhibitors of osteoclast differentiation and function improve bone mechanics and attenuate deterioration of bone architecture (56). BPs and denosumab are two commonly prescribed osteoclast inhibitors with distinct pharmacology (6). BPs, analogues of pyrophosphate, bind to bone mineral and upon resorption enter the intracellular compartment of osteoclasts, and induce apoptosis by interfering with vital cellular functions (12,180). Denosumab, a monoclonal antibody against RANKL, sequesters RANKL and inhibits osteoclast differentiation and function (98).

Although BPs and denosumab are designed to specifically target the osteoclasts, off-target effects for both agents have been reported (100,101,181-184). Oral BPs can induce gastrointestinal mucosal erosion and ulcer formation (185). BPs inhibit osteoblast apoptosis, but increase oral epithelial cell apoptosis *in vitro* (186), are taken up by bone marrow monocytes and dendritic cells, and are deposited in the perilacunar space around osteocytes (187). Denosumab, in addition to inhibiting bone resorption, may increase the risk for infections, as RANKL has been reported to be a co-stimulatory molecule for T cell activation (188). In mice, RANKL expression attenuates skin inflammation, suggesting that denosumab treatment could possibly enhance inflammatory responses on the skin (189). Furthermore, osteoprotegrin (OPG), the decoy receptor for the RANK pathway, is involved in muscle function; as such, denosumab treatment may affect muscle homeostasis (190).

Use of antiresorptive therapeutics can be associated with challenging side effects that can complicate treatment. Such side effects could be the direct result of the altered osteoclastic differentiation and function, or the indirect outcome of unwanted off-target actions. A prime example is ONJ. Indeed, hypotheses on ONJ pathophysiology include both direct and indirect effects of antiresorptives on the cellular infrastructure of the alveolar bone or the oral mucosa (103,191).

In the case of ONJ, understanding of the pathophysiology of the disease is further complicated by the fact that medications other than antiresorptives, such as anti-angiogenic and disease modifying antirheumatic drugs (DMARDs), are associated with similar oral complications. Thus, the term ONJ was introduced as an umbrella term to describe patients with jaw bone exposure under treatment by various classes of medications. The multiple cellular targets, potential off-target effects, and unwanted side effects of all these medications create a challenge in identifying common denominators that underlie the pathogenesis of jaw bone exposure. It is likely that despite similar clinical appearances, the pathophysiologic mechanism responsible for ONJ with use of each of these medications, and by extension the therapeutic approach that should be followed, would be distinct.

The occurrence of genetic diseases with known mutations, that show the same clinical features as those of a certain disease, can be valuable in understanding the pathogenesis of this disease. Here, we present three patients with such genetic diseases who also presented with ONJ. One patient suffered from osteopetrosis, while two patients suffered from pycnodysostosis. Both of

these genetic disorders impair osteoclast function (173). Osteopetrosis is a group of rare, inherited disorders, characterized by dysfunctional osteoclasts due to mutations in osteoclast machinery. Autosominal domininat osteopetrosis, the mildest form of the disease, is often due a mutation in the chloride channel gene, CLCN7, important in the acidification process of bone resorption (192). These patients often present with osteosclerosis and fractures of the long bones or ribs in late childhood or adulthood; however, they can often remain asymptomatic throughout life (192). Intermediate autosomal recessive osteopetrosis, also results from mutations in the CLCN7 gene, with increased bone density and fractures as the main symptoms (193). Autosomal recessive osteopetrosis, sometimes called malignant infantile osteopetrosis, is apparent from birth and can be fatal if untreated (194). Mutations in TCIRG1, which codes for a subunit of the vacuolar ATPase responsible for creating an acidic environment at the resorptive front, are commonly found in these patients (195). Patients suffer from vision loss, macrocephaly, hydrocephalus, hearing loss, and sometimes developmental delay (194,195). Finally, X-linked recessive osteopetrosis, an extremely rare form of the disease, is associated with immunodeficiency (173). Common dental findings in all patients with osteopetrosis are delayed tooth eruption and tooth impaction (173,196). Pycnodysostosis is a rare autosomal recessive mutation of the cathepsin K gene encoding for a lysosomal protease involved in the breakdown of bone organic matrix (197-199). Patients with pycnodysostosis demonstrate short stature, aplasia of the terminal phalanges, and show specific facial and oral manifestations, such as frontal and parietal bossing, dolichocephaly, narrow grooved palate, midfacial hypoplasia, obtuse mandibular gonial angle, micrognathia and hypodontia associated with multiple impacted teeth (200-202).

The patients presented here demonstrated genetic features of osteoclast dysfunction and none of them had been treated with medications known to be associated with ONJ, had received radiation to the jaws or had metastatic cancer. Clinical, radiographic, and histologic findings in these patients were identical to findings described in the literature for patients with ONJ. Radiographic changes in Patient 1 extended to the floor of the maxillary sinus, beyond the alveolar bone, consistent with a diagnosis of Stage 3 ONJ (1,2). Patient 2 presented with fistulae and evidence of infection, leading to a diagnosis of Stage 2 ONJ (1,2). Finally, Patient 3 presented with only exposed bone and was diagnosed with Stage 1 ONJ (1,2). Bone exposure in the patients followed tooth extraction (Patients 1 and 2) or dental disease (Patient 3), and was associated with various degrees of pain or signs of infection or inflammation. Non-healing extraction sockets or surgical sites with bone exposure are classic presentations of ONJ in patients on antiresorptive medications (Fig. 1).

Radiographic evaluation confirmed increased bone density with sequestration in areas of previously extracted teeth or around the lingual and buccal cortical plates, lytic defects and cortical erosions in all three patients. Non-healed extraction sockets were seen in two of the three patients. These radiographic findings are identical to that described in the literature for patients who have developed ONJ while receiving antiresorptive agents(167,170,203,204). Panoramic and CBCT images of two such patients are presented in Fig 2. There is a marked overlap of the radiographic features of the osteopetrotic and pycnodysostotic patients with those of the ONJ patients. Interestingly, impacted teeth were noted in the osteopetrotic and one of the pycnodysostotic patients. Impacted teeth are frequent findings in both diseases. Finally, both pycnodysostotic patients presented with short mandibular rami and obtuse mandibular angles, a

typical appearance of the mandibular shape in this patient population. The histologic findings (widespread areas of osteonecrosis, decreased bone remodeling, inflammatory infiltrate and bacterial colonization) were identical between the patients with osteopetrosis or pycnodysostosis and the patients who developed ONJ while on BPs or denosumab (Fig 3).

While, to the best of our knowledge, we are the first to use the term "ONJ" for the exposed necrotic bone in patients with osteopetrosis and pycnodysostosis, others have described similar findings. An early, insightful review of ONJ compared the clinical picture of osteonecrosis in patients with osteopetrosis to patients with ONJ and described them as "identical" while hypothesizing that BP associated ONJ is "a chemically induced form of osteopetrosis" (144). Exposure and infection of the alveolar bones were described in patients with osteopetrosis or pycnodysostosis nearly forty years prior to the first reports of ONJ (205). Publications of oral complications of patients with osteopetrosis consistently report sequestration, presence of necrotic alveolar bone exposed to the oral cavity, and failure of extraction socket healing (201,206-221). In the past, these findings were considered secondary to common jaw infections, and thus, were reported as osteomyelitis. Placing the clinical, radiographic and histologic findings of patients with pycnodysostosis and osteopetrosis reported in this paper and in the literature within the current understanding of ONJ points to a common etiologic mechanism. The development of ONJ in these patients, strongly suggests that these genetic disorders affecting osteoclast function have a common mechanism as ONJ in patients on antiresorptives: inhibition of osteoclast function and defective bone resorption.

Characterizing the oral complications of patients with osteopetrosis or pycnodysostosis as ONJ informs clinical management, that should adhere to the guidelines proposed by the ONJ literature

(1,2). Meticulous oral preventive measures, avoidance of tooth extractions or surgical trauma, if possible, and aggressive management of caries, periodontal, and periapical disease with short recall periods would be advised (1,2). It is noteworthy that 2 of our 3 patients did not have symptoms of ONJ until after routine tooth extractions, raising the issue whether these extractions could have been delayed, avoided or approached under non-traumatic surgical procedures. Although the number of patients included in our study is limited, our findings, in combination with the published body of work on ONJ and on other patients with osteopetrosis or pycnodysostosis lead us to propose a modified ONJ classification scheme (Fig. 18). Based on this classification, the ONJ definition should be amended, such that the first stipulation, as proposed in the AAOMS position paper (1,2), defines patients to be considered to have ONJ if "1. Current or previous treatment with antiresorptive or antiangiogenic agents", *or a genetic condition that affects osteoclast function* is present. The term ONJ should continue to be used since it has clinical usefulness, particularly as new medications with an ONJ-like phenotype continue to be identified. We introduce the term Osteoclast Related ONJ (ORONJ) to describe patients with ONJ, where deficient osteoclastic function, either pharmacologically or genetically induced, is present. The term "ORONJ" provides a pathophysiologic definition which offers definite advantages, as it: 1) identifies osteoclasts as the target cell, implying a clear causal relationship between the medication or genetic defect and the clinical presentation; 2) predicts that new medications that directly target osteoclastic differentiation and function could induce ONJ; 3) informs and targets the design of new therapeutic interventions; 4) allows the oral complications of patients with osteopetrosis and pycnodysostosis to be diagnosed as ONJ with implications for clinical management, and; 5) distinguishes between BPs or denosumab vs other medications that are associated with ONJ. Under current understanding of the disease and

epidemiologic clinical data, most patients with ONJ should be classified as suffering from the ORONJ form of ONJ. It remains to be explored whether all forms of ONJ have a common biologic framework or if they represent phenotypically similar, but pathophysiologically distinct, entities.

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AUTHORS' ROLES

Study design: DH, AS, SMD, PHK, TLA, and ST. Study conduct: DH, TLA, and ST. Data analysis: DH, AS, SMD, PHK, TLA, and ST. Data interpretation: DH, AS, SMD, PHK, TLA, and ST. Drafting manuscript: DH, TLA, and ST. Critically revising manuscript: AS, SMD, PHK. Approving final version of manuscript: DH, AS, SMD, PHK, TLA, and ST. All authors had full access to data and take responsibility for the integrity of the data and accuracy of the data analysis.

FIGURES

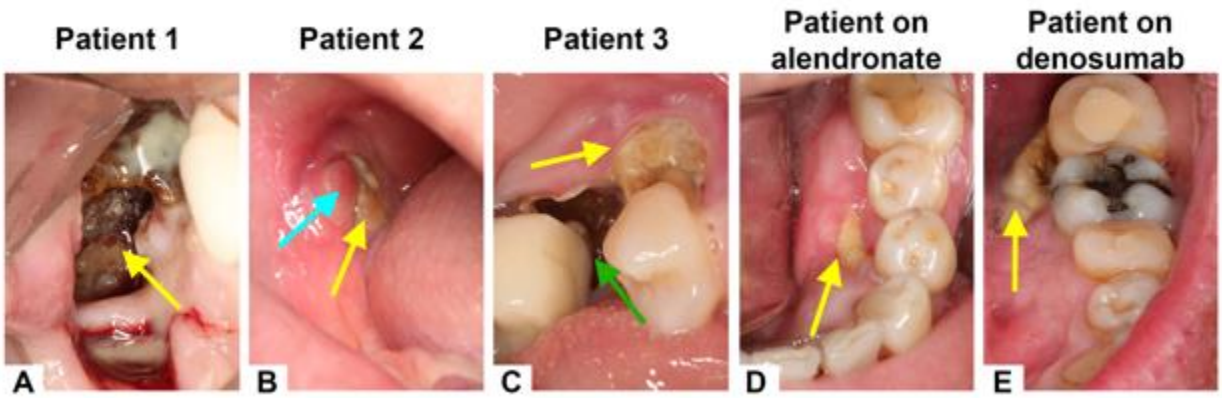

Figure 14: Clinical Presentation of Patients

A) Clinical image of Patient 1 with osteopetrosis. Yellow arrow points to an area of exposed bone in the maxillary right quadrant associated with previous history of tooth extraction. **B)** Clinical image of Patient 2 with pycnodysostosis. Yellow arrow points to bone exposure in the right posterior mandible while soft tissue edema and erythema is noted (cyan arrow). **C)** Clinical image of Patient 3 with pycnodysostosis. Yellow arrow points to bone exposure on the buccal surface of maxillary right premolar. A tooth fracture is present (green arrow), while no significant erythema is noted. **D)** Clinical image of a patient with Stage 1 ONJ treated with alendronate. Yellow arrow points to an area of exposed bone on the lingual surface of the left mandible. **E)** Clinical image of a patient with Stage 2 ONJ treated with denosumab. An area of exposed bone on the alveolar and lingual surfaces of a patient taking denosumab who had his mandibular left third molar extracted 6 months ago. Yellow arrows point to areas of bone exposure.

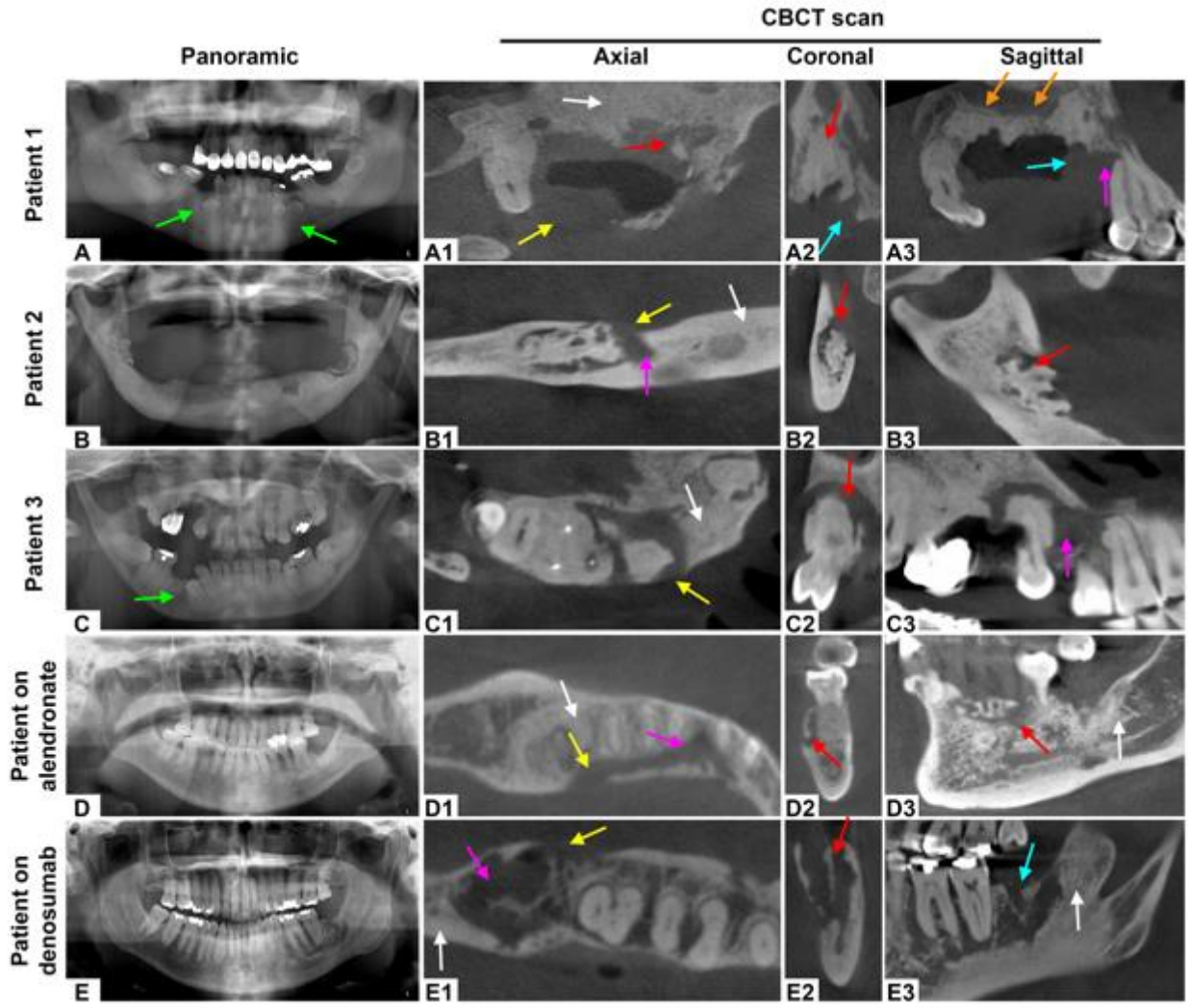

Figure 15: Radiographic Findings of Patients

Panoramic radiographs, and axial, coronal, and sagittal CBCCT sections of Patient 1 with osteopetrosis **(A, A1, A2, A3)**, Patient 2 with pycnodysostosis **(B, B1, B2, B3)**, Patient 3 with pycnodysostosis **(C, C1, C2, C3)**, an ONJ patient on alendronate **(D, D1, D2, D3)**, and an ONJ patient on denosumab **(E, E1, E2, E3)**. Green arrows point to impacted teeth, white arrows to areas of increased trabecular bone density, red arrows to sequestration, yellow arrows to cortical erosion, cyan arrows to unhealed extraction sockets, orange arrows to disrupted maxillary sinus floor and purple arrows to lytic changes.

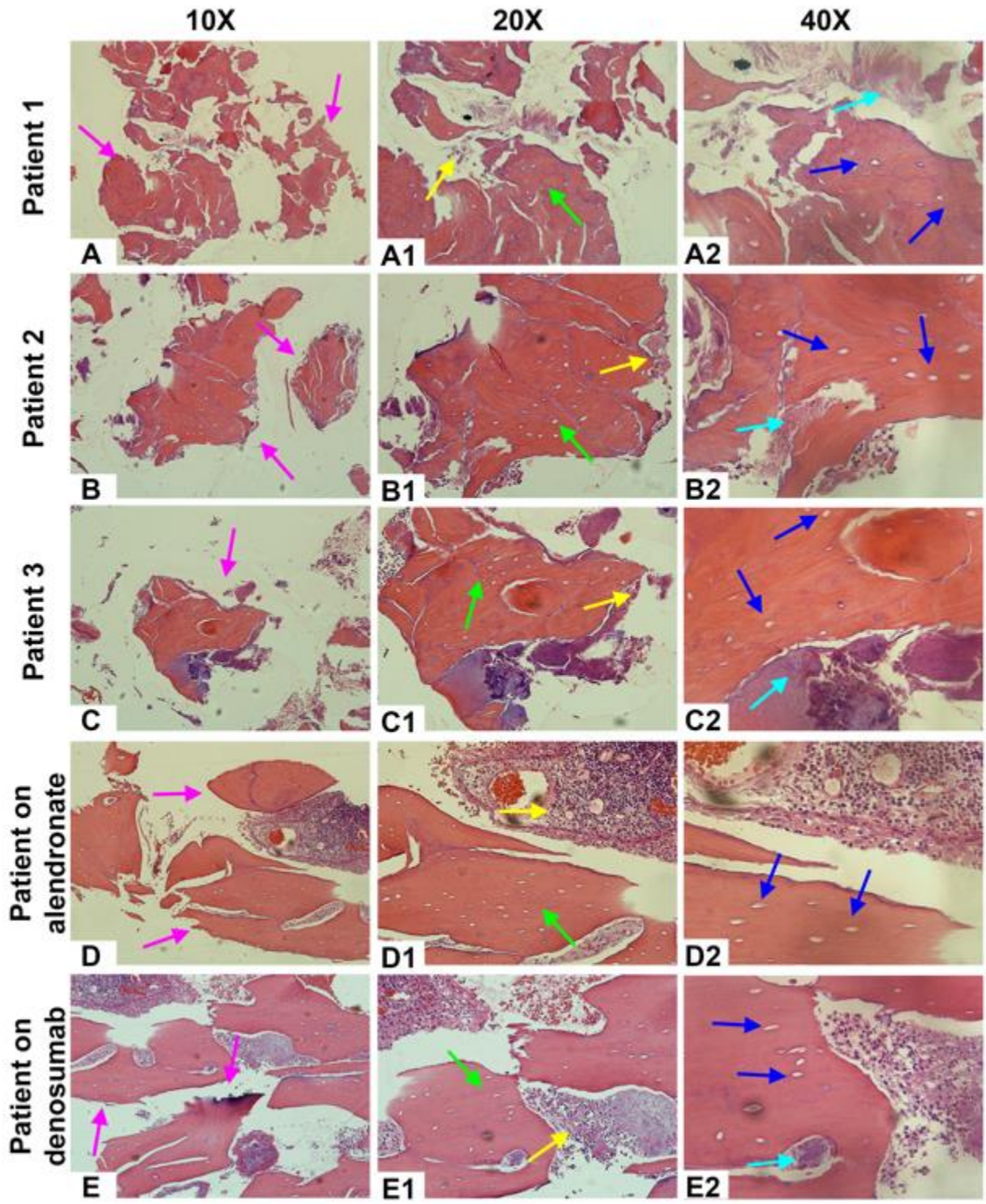

Figure 16: Histologic analysis

10x, 20x, and 40x photomicrographs, of histologic samples obtained from Patient 1 with osteopetrosis **(A, A1, A2)**, Patient 2 with pycnodysostosis **(B, B1, B2)**, Patient 3 with

pycnodysostosis **(C, C1, C2)**, an ONJ patient on alendronate **(D, D1, D2)**, and an ONJ patient on denosumab **(E, E1, E2)**. Purple arrows point to areas of sequestra, yellow arrows to areas of acute and chronic inflammation, green arrows to large areas of osteonecrosis, blue areas to empty osteocytic lacunae, and cyan arrows to bacterial colonization.

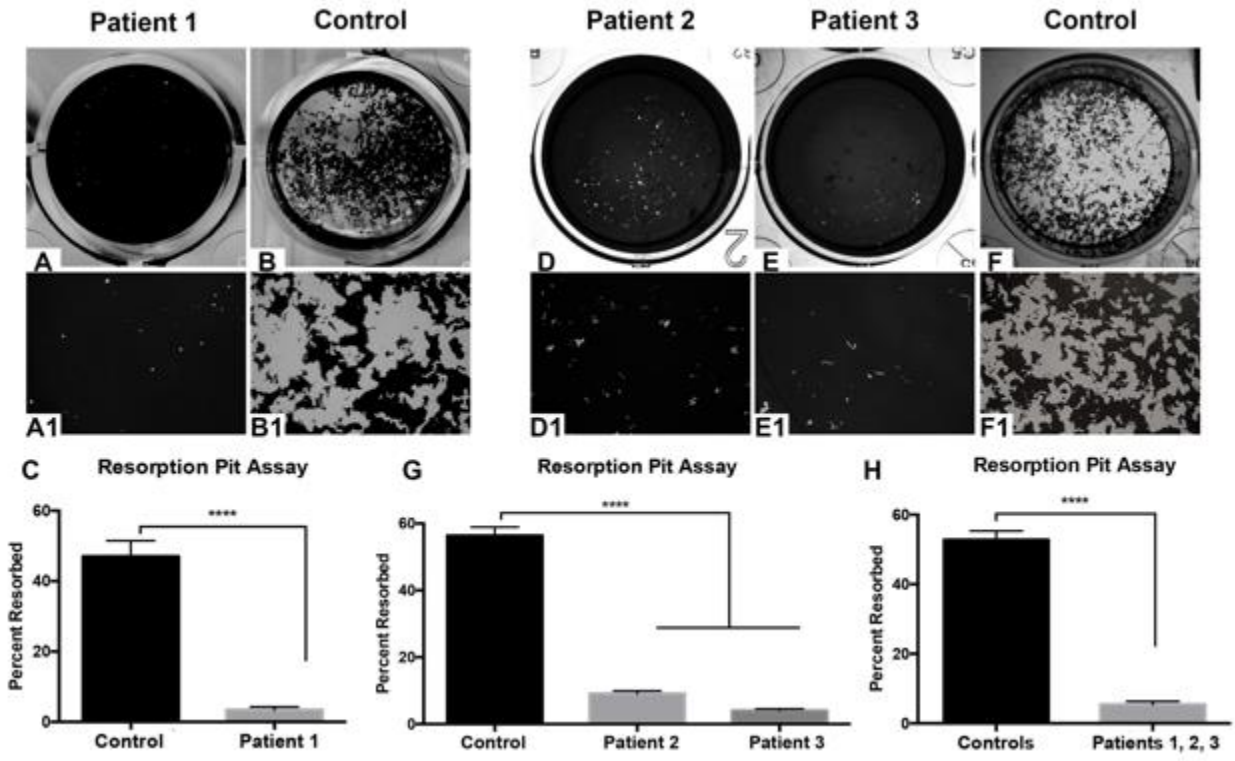

Figure 17: In-Vitro Resorption Assays of Osteoclasts Differentiated from PBMC's

Representative wells from the resorption pit assay of PBMCs from Patient 1 with osteopetrosis **(A, A1)** or age, gender matched controls **(B, B1)** and quantification of the resorptive area **(C)**. Representative wells from the resorption pit assay of PBMCs from Patient 2 with pycnodysostosis **(D, D1)**, from Patient 3 with pycnodysostosis **(E, E1)** or age, gender matched controls **(F, F1)** and quantification of the resorptive area **(G)**. Quantification of the resorptive area of all patients and all controls **(H)**. ****: statistically significant with $p<0.0001$

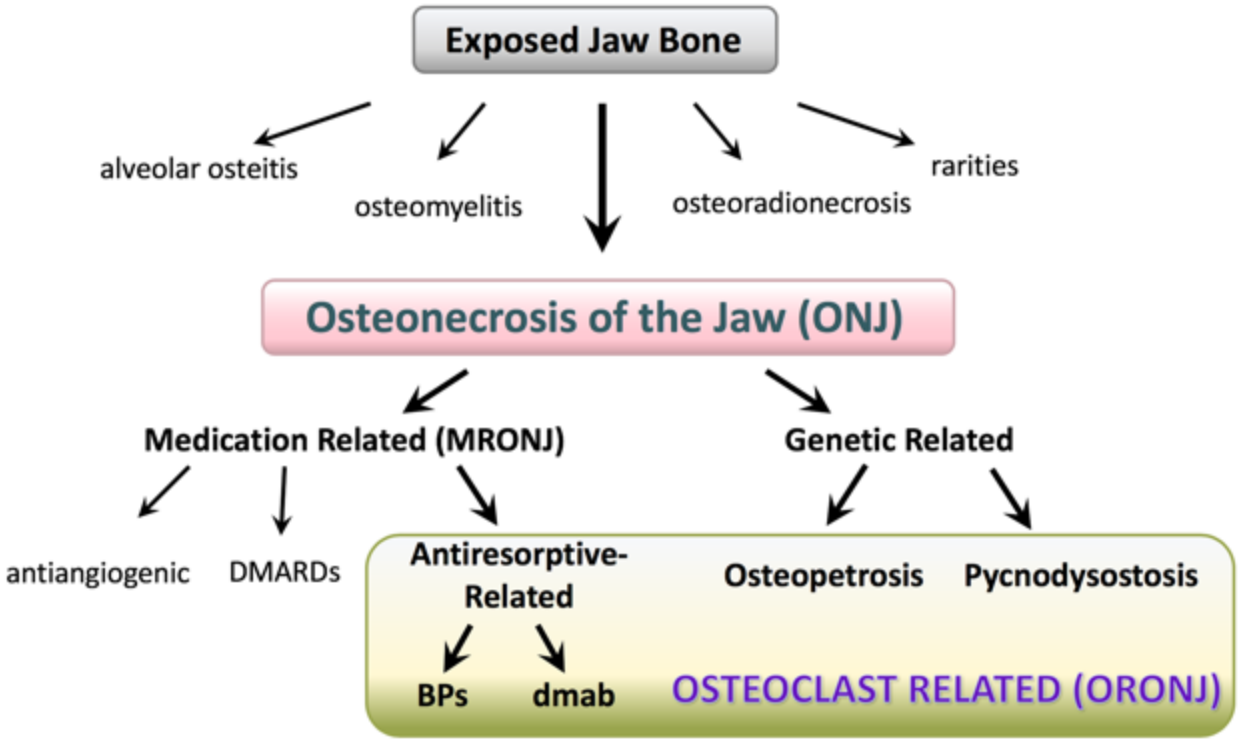

Figure 18: Proposed Model of ONJ classification

Bone exposure in the oral cavity is the result of osteonecrosis of the jaw (ONJ), alveolar osteitis, osteomyelitis, osteoradionecrosis or other rarer conditions. ONJ could be the side effect of various medications that patients receive for management of a systemic disease (ONJ) or the presentation of a genetic condition. Osteoclasts play a pivotal role in a subset of ONJ cases in patients receiving osteoclast inhibitors, as well as in genetic conditions that alter osteoclastic function and/or differentiation, such as osteopetrosis or pycnodysostosis. Thus, we propose the use of the term osteoclast related ONJ (ORONJ) to differentiate between these patients where the target cell is the osteoclast directly versus other ONJ patients with different cellular targets.

Chapter 6: Discontinuation of OPG-Fc, but not ZA, Prior to Tooth Extraction Ameloriates Osteonecrosis of the Jaw (ONJ) in Rats

ABSTRACT

Osteoporosis and bone malignancies are conditions characterized by an increase in osteoclastic bone resorption. Although their pathogenesis is distinct, both diseases are commonly treated with antiresorptives, such as bisphosphonates and denosumab. Bisphosphonates are analogues of naturally occurring pyrophosphate molecules. Deposited into areas of active bone resorption, they lead to apoptosis when uptaken by osteoclasts during bone resorption. Similarly, denosumab, a monoclonal antibody against the receptor activator of nuclear kappa-beta ligand (RANKL), prevents osteoclast differentiation and function by binding to and sequestering circulating RANKL. Despite two distinct mechanisms of action, both medications are associated with osteonecrosis of the jaw (ONJ), an oral complication characterized by areas of non-healing exposed bone in the oral cavity. Once developed, treatment options range from treatment of symptomology to surgical resection of the affected area. With minimal treatment options, disease prevention becomes exceedingly important. Oral trauma, in the form of tooth extraction, is the most common local instigating factor in ONJ development. As such, the idea of antiresorptive discontinuation around times of dental treatment has been proposed; however, scant evidence exists to support its use. Here, we investigate the ability of the discontinuation of zoledronic acid (ZA), a potent bisphosphonate, and OPG-Fc, a denosumab surrogate, discontinuation to mitigate ONJ development in rats. We report that discontinuation of either medication after ONJ establishment did not improve ONJ resolution. However, discontinuation of OPG-Fc, but not ZA, prior to tooth extraction ameliorates ONJ development.

INTRODUCTION

Antiresorptive medications, such as bisphosphonates (BPs) and denosumab, are commonly used for the management of osteoporosis and bone malignancies (56,222). Despite their clinical efficacy at managing osteoporotic complications and minimizing skeletal related events, these medications are associated with infrequent, but serious complications, such as atypical femur fractures and Osteonecrosis of the Jaws (ONJ) (101,223). ONJ, initially described in 2003 and 2004, is defined as exposed bone in the maxillofacial region that lasts for at least 8 weeks, in the presence of anti-resorptive therapy, without a history of radiation (1-4).

Clinical and translational findings have identified that systemic antiresorptive treatment, in conjunction with oral trauma, such as tooth extraction, or periodontal and periapical disease are often associated with ONJ development (2,62,66,77). As a result, dental management of patients on antiresorptive medications, particularly those that require surgical intervention, can be clinically challenging. (2,47). To minimize ONJ risk, discontinuation of antiresorptive administration has been proposed. Position papers by both the AAOMS and ASBMR propose guidelines for antiresorptive discontinuation, acknowledging that minimal to no data exist to support the use, and any recommendations provided are largely empirical (1,2). Furthermore, drug discontinuation is not without risks, as it may potentially lead to skeletal complications from the systemic disease (224-226). Given that ONJ is a rare disease, clinical trials to address the usefulness of antiresorptive discontinuation would be difficult. As such, the investigation of antiresorptive discontinuation in pre-clinical animal models becomes of utmost importance.

Previous studies by our group, and others, have identified that systemic antiresorptive therapy combined with periodontal or periapical disease, induce ONJ-like lesions in the alveolar bone of rodents. Using a model of periapical disease, we have demonstrated that discontinuation of the RANK-L inhibitor, OPG-Fc, but not of the potent bisphosphonate, zoledronic acid (ZA), reverses ONJ in mice (78). These findings are clinically relevant, particularly in patients with spontaneous ONJ developing around existing teeth with periodontal or periapical disease. However, most cases of ONJ occur after local trauma, and particularly tooth extraction. We have also reported that extraction of teeth with experimental periapical disease induces ONJ-like lesions that clinically, radiographically and histologically mimic human ONJ lesions (35). These studies emphasize the role of pre-existing dental disease with subsequent dental extraction for ONJ development and provide an appropriate translational model to explore the role of antiresorptive discontinuation in the healing of extraction sockets, and potential development of ONJ.

Here, utilizing a well-defined, reproducible ONJ model of tooth extraction (35), we investigate the effect of antiresorptive discontinuation on ONJ development in two specific settings. In the first, we investigate whether antiresorptive withdrawal before tooth extraction mitigates ONJ development. In the second setting, we examine if antiresorptive withdrawal after established ONJ can lead to resolution of the disease. We report that withdrawal of OPG-Fc, but not ZA, before tooth extraction ameliorated ONJ development. However, discontinuation of either OPG-Fc or ZA after tooth extraction, did not reduce the incidence or burden of ONJ-like lesions.

MATERIALS AND METHODS

Animal care

This study was approved by the UCLA Chancellor's Animal Research Committee (ARC). Animals were housed following the ARC guidelines. Two-month-old male Wistar Han rats (Charles River Laboratories, Raleigh, NC) (1 per cage) were boarded in pathogen-free conditions with a 12-hour light/dark cycle. A standard diet (NIH-31 Modified Open Formula, ENVIGO, Madison, WI, USA) and water were provided *ad libitum*. A randomized, controlled, animal model design was utilized for this prospective study, following all the recommendations of the Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines. Investigators performing analysis were blinded to treatment groups.

Animals received intraperitoneal (IP) injections of Veh (endotoxin-free saline), 200 µg/kg Zoledronic Acid (LKT Laboratories, St Paul, MN), or 10mg/kg OPG-Fc (composed of the RANKL-binding domains of osteoprotegerin linked to the Fc portion of IgG) twice weekly.

Experimental Analysis: Ex-vivo µCT specimen scanning & Imaging

Following the end of the experimental period, mandibles were harvested and imaged using a digital microscope at 40x magnification (Keyonce VHX-100, Osaka, Japan). Mandibles were fixed for 48 hours in 4% paraformaldehyde, and imaged by ex vivo micro-computed tomography (µCT) using SkyScan 1172 at 20µm resolution (SkyScan, Kontich, Belgium), as previously described (35). All animals that were detected to have retained root tips in the areas of the extraction sockets were excluded from any further analysis.

Histologic Analysis and TRAP staining

Mandibles were decalcified in 15% EDTA and sectioned in a buccal-lingual fashion, in the area of bone exposure. Samples were paraffin embedded and 5μm sections were made and stained with H&E (38). Analysis was performed using Aperio Image Scope software (Aperio Technologies, Inc., Vista, CA). Histology and digital scanning of the slides was performed at the Translational Pathology Core Laboratory (TPCL) at the David Geffen School of Medicine at UCLA. The region of interest (ROI) was defined as the area of the alveolar crest to the inferior border of the mandible in the area of M1 and M2. The epithelium to alveolar crest distance, total number of osteocytic lacunae, number of empty lacunae, and osteonecrotic area were quantified (38). Empty osteocytic lacunae were those with missing or karolytic osteocytes, as described previously (46). Osteonecrosis was identified as an area of 5 or more confluent empty lacunae. The epithelium to alveolar crest distance was the distance from the inferior part of the epithelium to the alveolar crest.

For osteoclast enumeration, tartrate-resistant acid phosphatase (TRAP) staining was performed utilizing the leukocyte acid phosphatase kit (387A-IKT Sigma, St. Louis, MO) and normalized to the bone surface area (66).

Experimental Design: Antiresorptive Discontinuation Before Tooth Extraction (Fig. 19A)

Rats were randomly assigned to receive two weekly injections of Veh, ZA, or OPG-Fc. Sixteen animals received Veh, 32 animals received ZA, and 32 animals received OPG-Fc. Animals were pre-treated for 1 week, after which experimental periapical disease (EPD) was induced, as previously described (35). Briefly, the pulp chamber of the first and second mandibular molars

(M1 and M2, respectively) was exposed and inoculated with periapical pathogens. Treatment continued for 3 weeks, after which ZA or OPG-Fc was discontinued in 16 rats of the ZA *(dZA)* and 16 OPG *(dOPG)* groups. The remaining 16 animals of the ZA (cZA) and 16 animals of the OPG (cOPG) groups continued to receive antiresorptive treatment. 1 week after antiresorptive discontinuation, the right M1 and M2 were extracted. The animals were euthanized 4 weeks after tooth extraction. Upon radiographic assessment, all animals with retained root tips were excluded from analysis. Eleven Veh, eight cOPG-Fc, eight dOPG-Fc, ten cZA, and nine dZA were included in the analysis.

Experimental Design: Antiresorptive Discontinuation After Tooth Extraction (Fig. 19B)

Rats were randomly assigned to receive two weekly injections of Veh, ZA, or OPG-Fc. Sixteen animals received Veh, 32 animals received ZA, and 32 animals received OPG-Fc. Animals were pre-treated for one week, after which EPD was induced, as above (35). Four weeks after induction of EPD, the right M1 and M2 were extracted. Antiresorptive treatment continued for 4 weeks, then ZA or OPG-Fc was discontinued in 16 rats of the ZA *(dZA)* and 16 OPG-Fc *(dOPG-Fc)* groups. The remaining 16 animals of the ZA (cZA) and 16 animals of the OPG-Fc (cOPG-Fc) groups continued to receive antiresorptive treatment. The animals were euthanized four weeks after antiresorptive discontinuation. Upon radiographic assessment, all animals with retained root tips were excluded from analysis. Twelve Veh, eleven cOPG-Fc, nine dOPG-Fc, thirteen cZA, and twelve dZA were included in the analysis.

Statistics

Raw data were analyzed using GraphPad Prism (GraphPad Software, Inc. La Jolla, CA). Descriptive statistics were used to calculate the mean and the standard error of the mean (SEM). Data were analyzed by two-way ANOVA and post-hoc Tukey's test for multiple comparisons, with statistical significance of 0.05. Socket healing was analyzed using the Fischer's exact test.

RESULTS

Discontinuation of ZA or OPG-FC Before Tooth Extraction (Fig. 19A)

Clinical assessment of the alveolar ridge demonstrated that nearly all (10/11 or 91%) Veh treated animals healed uneventfully with normal mucosal coverage (Fig. 20A, F). In contrast, 88% (7/8) cOPG-Fc treated animals presented with exposed bone and mucosal defects (Fig. 20B, F). Upon discontinuation of OPG-Fc for 1 week before tooth extraction (dOPG-Fc), the great majority of animals (7/8 or 88%) demonstrated normal socket healing and mucosal coverage (Fig. 20C, F). Similar to cOPG-Fc animals, 70% (7/10) cZA treated animals presented with bone exposure and ONJ-like lesions (Fig. 20D, F). Interestingly, in the dZA group, the majority of the animals (6/9 or 67%) presented with bone exposure and mucosal defects (Fig. 20E, F).

Radiographically, Veh treated animals showed osseous healing and remodeling of the socket outline, such that the extraction sockets could not be distinguished from the adjacent alveolar ridge. Woven bone occupied the entirety of the remodeled ridge (Fig. 21A-A2). In cOPG-Fc and cZA treated animals, lack of socket healing was observed with the outline of the extraction socket clearly visible (Fig. 21B-B2, D-D2, yellow arrows). Prominent periosteal bone formation (Fig. 21B-B2, D-D2, cyan arrows) was noted on the buccal and lingual cortices of these animals. Similarly, in the dZA group, extraction sockets remained poorly remodeled, and appeared very similar to animals without antiresorptive withdrawal (Fig. 21E-E2). In contrast, in animals treated with OPG-Fc that was discontinued 1 week before extraction (dOPG-Fc), the alveolar ridge appeared remodeled, and the extraction sockets could no longer be distinguished from the remaining alveolar bone (Fig. 21C-C2).

Histologic investigation revealed that Veh and dOPG-Fc treated animals had normal epithelium with rete pegs, normal submucosa void of prominent inflammatory infiltrate, low levels of empty osteocytic lacunae and no areas of osteonecrosis (Fig. 22 A-A1, C-C1, green arrows). In contrast, cOPG-Fc, cZA, and dZA animals showed exposed bone with disrupted epithelium, debris, strong inflammatory infiltrate, high levels of empty osteocytic lacunae and areas of osteonecrosis (Fig. 22 B-B1, D-D1, E-E1, yellow and white arrows). Quantification of histologic findings revealed statistically significant differences of Veh and dOPG-Fc vs cOPG-Fc, cZA or dZA in empty osteocytic lacunae, osteonecrotic area, and epithelial to crest distance (Fig. 22 F-H). Finally, the number of osteoclasts was significantly increased in dOPG-Fc animals, when compared to all remaining groups (Fig. 22I).

Withdrawal of ZA or OPG-FC After Tooth Extraction (Fig. 19B)

Clinical evaluation of extraction sockets in Veh animals revealed normal mucosa with uninterrupted healing in 92% (11/12) Veh treated animals (Fig. 23A, F). In contrast, 73% (8/11) cOPG-Fc and 66% (6/9) dOPG-Fc animals presented with bone exposure (Fig. 23B, C, F). Similarly, 77% (10/13) and 66% (8/12) of cZA and dZA animals presented with bone exposure (Fig. 23D, E, F). No statistical differences in bone exposure were noted among treated groups (Fig. 23F), which were all statistically different vs. Veh control.

Radiographic evaluation revealed complete socket remodeling in Veh treated animals (Fig. 24A-A2). In all remaining groups, extraction socket outlines remained distinct, and partial or complete absence of bone formation was noted within the extraction sockets (Fig. 24A-A2, B-B2, C-C2, D-D2, E-E2, yellow arrows). Prominent periosteal bone formation (Fig. 24A-A2, B-

B2, C-C2, D-D2, E-E2, cyan arrows) was noted on the buccal and lingual cortices of these animals.

Histologic evaluation in Veh animals revealed mucosal healing, with healthy epithelium with rete peg formation, normal submucosa void of prominent inflammatory infiltrate, and lack of empty osteocytic lacunae and of osteonecrotic areas (Fig. 25A-A1). Osteoclasts were present in contact with the alveolar bone. In contrast, most animals in the cOPG-Fc and dOPG-Fc groups presented with exposed bone, disrupted epithelium with debris collection in the remnants of the extraction sockets (Fig. 25B-B1, C-C1). Osteoclasts were largely absent in both groups. Similarly, in the ZA and dZA animals, exposed bone was present with prominent inflammatory infiltrate and areas of osteonecrosis (Fig. 25D-D1, E-E1). Similar to the cOPG-Fc and dOPG-Fc groups, osteoclasts were largely absent, with similar epithelial-alveolar crest distance (Fig 24H, I). No statistical differences in any variables were noted among groups receiving cOPG-Fc, dOPG-Fc, cZA, or dZA (Fig. 25 I-H). All groups were statistically significantly different vs Veh control animals (Fig. 25 I-H).

DISCUSSION

ONJ can be challenging to manage, with treatment options ranging from non-surgical management to surgical intervention, often involving major reconstruction [47,48,50,227]. Antiresorptive medications associated with ONJ include BPs and denosumab, a humanized monoclonal antibody against RANKL. Our findings here provide translational evidence that drug discontinuation, in specific settings, could be used to ameliorate ONJ development.

BPs, potent analogues of inorganic pyrophosphate, bind hydroxyapatite crystals, and show high affinity for bone mineral (12). Upon administration, BPs are integrated into areas of active bone turnover, while unincorporated drug is rapidly excreted (228). During bone resorption, hydroxyapatite-bound BPs are internalized in actively resorbing osteoclasts (229,230). Non-nitrogenous BPs, such as etidronate and clodronate, are converted into non-functional ATP analogues intracellularly, where they accumulate in high concentrations and induce osteoclast apoptosis (231). In addition, nitrogen containing BPs, such as alendronate and zoledronate, inhibit farnesyl diphosphate synthase, and decrease synthesis of geranylgeranyl and farnesyl diphosphate, moieties required for prenylation of proteins, such as small GTPases. Disruption of these pathways leads to osteoclast apoptosis (232). Bone mineral bound BPs exert their effects long after their administration (233). The half-life of these drugs remains a subject of debate, but a skeletal half-life of more than 10 years in those receiving intravenous fusions has been suggested (19,233).

Denosumab, a monoclonal antibody against RANKL, approved for the treatment of osteoporosis and bone metastases, inhibits bone resorption by preventing differentiation and maturation of osteoclasts (98). Osteoprotegrin (OPG) and RANKL, major regulators of osteoclastogenesis, are tightly controlled in homeostasis. RANKL binds to and activates the RANK receptor on the surface of osteoclast precursors and mature osteoclasts, leading to osteoclast differentiation (234,235). In contrast, OPG acts as a decoy receptor that binds RANKL and interferes with RANK activation, thus preventing osteoclast differentiation. Similarly, denosumab sequesters circulating RANKL, mimicking the natural function of OPG, and ultimately preventing osteoclast

differentiation (18). Different from BPs, denosumab availability is almost immediate, detectable within 1 hour of subcutaneous delivery. Highest serum concentrations are achieved 5-21 days after administration, with a drug half-life of 32 days (7,236).

Denosumab, a humanized antibody, does not recognize mouse or rat RANKL. As a substitute, rat OPG-Fc, composed of the RANKL-binding domains of OPG linked to the Fc portion of IgG, has been used to block RANKL activity in mice (237-242). Indeed, OPG-Fc successfully decreases tumor burden and increases survival in mice with multiple myeloma, prevents tumor induced bone resorption in mice with estrogen receptor positive breast cancer, and can enhance the anti-tumor effects, preventing osteolysis, in mice with epidermoid carcinoma bone metastasis (237-240,242). Additionally, OPG-Fc has been used to prevent ovariectomy induced bone loss in mice (241). BPs, such as zoledronic acid, have also been used to prevent the spread of bone metasasis in mice with multiple myeloma (243). We have previously shown the dose of OPG-Fc used here completely eliminates all osteoclast function (78). Importantly, both antiresorptives have provided similar incidence and burden of ONJ (37,46,78).

Due to the distinct bioavailability and pharmacologic mode of function, osteoclast recovery is disparate under discontinuation of BP or denosumab treatment (244,245). From an oral management perspective, antiresorptive discontinuation becomes relevant in two specific clinical settings. In the first setting, a patient on antiresorptive medications presents with a non-restorable tooth that requires extraction. Although tooth extraction is the most common local instigating factor for ONJ, it often is the only way to remove the local infection/inflammation. Such a patient could potentially benefit from discontinuation of the antiresorptive medication prior to tooth extraction

in an effort to recover the local remodeling capacity of the alveolar bone. The second scenario involves a patient on antiresorptives with bone exposure and clinical ONJ. For such a patient, antiresorptive discontinuation could potentially improve mucosal and hard tissue healing. Although tooth extraction in patients on antiresorptives or management of patients with ONJ can pose significant challenges, little clinical or translational evidence exists to support antiresorptive discontinuation for the management of these patients (1,2).

To provide insight in these clinical settings, we employed a previously described ONJ model that utilizes experimental periapical disease and tooth extraction to develop ONJ-like lesions in rats treated with high dose antiresorptives (35). Using this model, in a first experimental approach animals received ZA or OPG-Fc, which was then discontinued prior to tooth extraction. Control animals healed uneventfully, as expected. On the other hand, the majority of animals that received continuous antiresorptive treatment developed ONJ-like lesions. The severity and prevalence of ONJ was similar between ZA and OPG-Fc treatment. Animals in which ZA was discontinued demonstrated a similar appearance, with clinical bone exposure, mucosal inflammation, lack of boney healing, and histologic osteonecrosis. In contrast, most animals with OPG-Fc discontinuation showed mucosal healing, with osseous remodeling, and reduction of histologic osteonecrosis. In a second experimental approach, we investigated the effects of antiresorptive withdrawal after tooth extraction and ONJ development. In this setting, withdrawal of either antiresorptive did not improve the healing of the extraction socket and established ONJ lesions. Clinical, radiographic and histologic assessment demonstrated that while control animals never developed ONJ lesions, similar ONJ burden was noted in all antiresorptive treated groups irrelevant of the continuous or discontinuous administration.

Our findings suggest that in patients on antiresorptives, if the management of the systemic disease allows, drug discontinuation should be considered in patients receiving denosumab prior to tooth extraction, since in these patients improved mucosal and osseous socket healing and decreased ONJ incidence could be achieved. In patients receiving BPs, either prior to tooth extraction or with established ONJ lesions, or in patients receiving denosumab with established ONJ lesions, antiresorptive discontinuation would not likely improve the oral tissue response. In such patients, management of the systemic disease should remain the primary focus.

We have previously reported on the discontinuation of antiresorptives in the presence of established ONJ lesions, but in the absence of tooth extraction (78). In these cases of ONJ around periapical or peri-radicular disease, OPG-Fc discontinuation reversed the radiographic and histologic features, while discontinuation of ZA did not. These findings diverge from our current observations where OPG-Fc discontinuation did not lead to the resolution of the ONJ lesions. There are significant differences in the experimental setting of the two studies that could explain this discrepancy. In our previous study in mice (78), ONJ was induced by either experimental or spontaneous dental disease without tooth extraction. This resulted in small areas of bone exposure that were not appreciated clinically, but were noted histologically. In contrast, in our present report in rats, experimental dental disease was induced and then teeth were extracted. This intervention resulted in large areas of clinical bone exposure readily observed by clinical inspection. An additional significant difference is that in our previous study discontinuation of antiresorptives extended for six and ten weeks, with recovery of radiographic and histologic indices of ONJ being more prominent at ten weeks. In contrast, in our current study the

discontinuation lasted only four weeks. This combination of smaller ONJ lesions and longer discontinuation times in our earlier study, probably resulted in a more effective removal of osteonecrotic areas and subsequent soft tissue healing.

The effects of ZA withdrawal for a total of 1-4 months before extraction of healthy teeth followed by an additional 2 months after bilateral tooth extraction was investigated and a decrease in bone exposure and osteonecrosis was reported (246). These findings disagree with our current and previously published data (78), where ZA withdrawal in various settings never improved osseous of soft tissue healing and ONJ burden. Distinct technical experimental methodologies including ZA dosage, cessation period, treatment period, and animal models could account for these disparities.

In conclusion, our data provide translational evidence that the discontinuation of the denosumab surrogate, OPG-Fc, but not the bisphosphonate, ZA, prior to tooth extraction ameliorates ONJ. At the clinical setting, other effects of antiresorptive discontinuation must be considered that were not tested in our studies. A documented effect of denosumab discontinuation is an increased risk of rebound associate vertebral fractures (226,247,248). The low frequency of ONJ in 0.01% of osteoporotic individuals could potentially make drug discontinuation unnecessary (1,2). In patients taking oncologic doses of antiresorptives, discontinuation should be primarily dictated by control of the underlying malignancy, which largely outweighs the decreased ONJ risk. Nevertheless, in appropriate individuals with control of their systemic disease, denosumab withdrawal could be effective in reducing the risk of ONJ development.

ACKNOWLEDGEMENTS

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AUTHORS' ROLES

Study design: DH, AS, TLA, and ST. Study conduct: DH, AS, IG, OB, TLA, and ST. Data analysis: DH, AS, IG, SMD, TLA, and ST. Data interpretation: DH, AS, IG, OB, SMD, FQP, TLA, and ST. Drafting manuscript: DH and ST. Critically revising manuscript: AS, IG, OB, SMD, FQP, and TLA. Approving final version of manuscript: DH, AS, IG, OB, SMD, FQP, TLA, and ST. All authors had full access to data and take responsibility for the integrity of the data and accuracy of the data analysis.

FIGURES

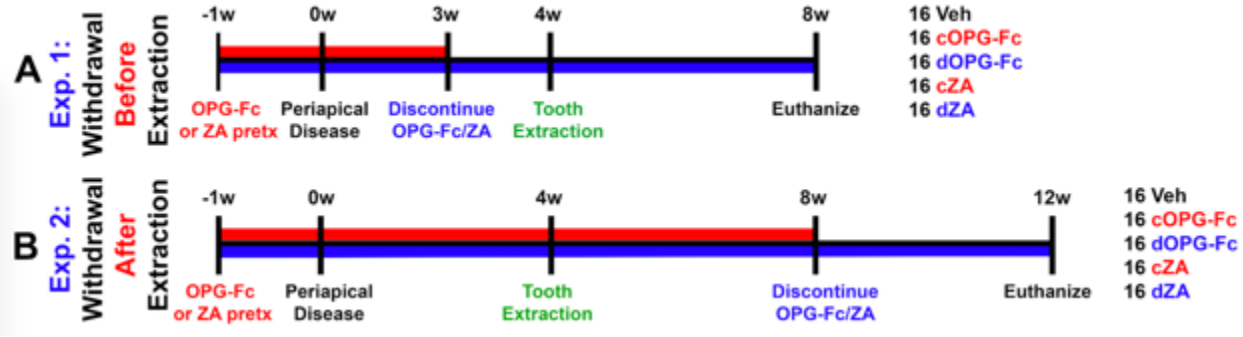

Figure 19: Experimental Design

(A) Experiment 1: Withdrawal of OPG-Fc and ZA Before Extraction: Animals were pretreated for 1 week with OPG-Fc or ZA. After 1 week of pretreatment, periapical disease was induced. 3 weeks after periapical disease and 4 weeks after treatment induction, OPG-Fc or ZA were discontinued in half of the animals. 1 week after discontinuation, tooth extraction was performed. 4 weeks after tooth extraction, animals were euthanized.

(B) Experiment 2: Withdrawal of OPG-Fc and ZA After Extraction: Animals were pretreated for 1 week with OPG-Fc or ZA. After 1 week of pretreatment, periapical disease was induced. 4 weeks after periapical disease was induced, tooth extraction was performed. 4 weeks after tooth extraction, OPG-Fc or ZA were discontinued in half of the animals. 4 weeks after discontinuation, animals were euthanized.

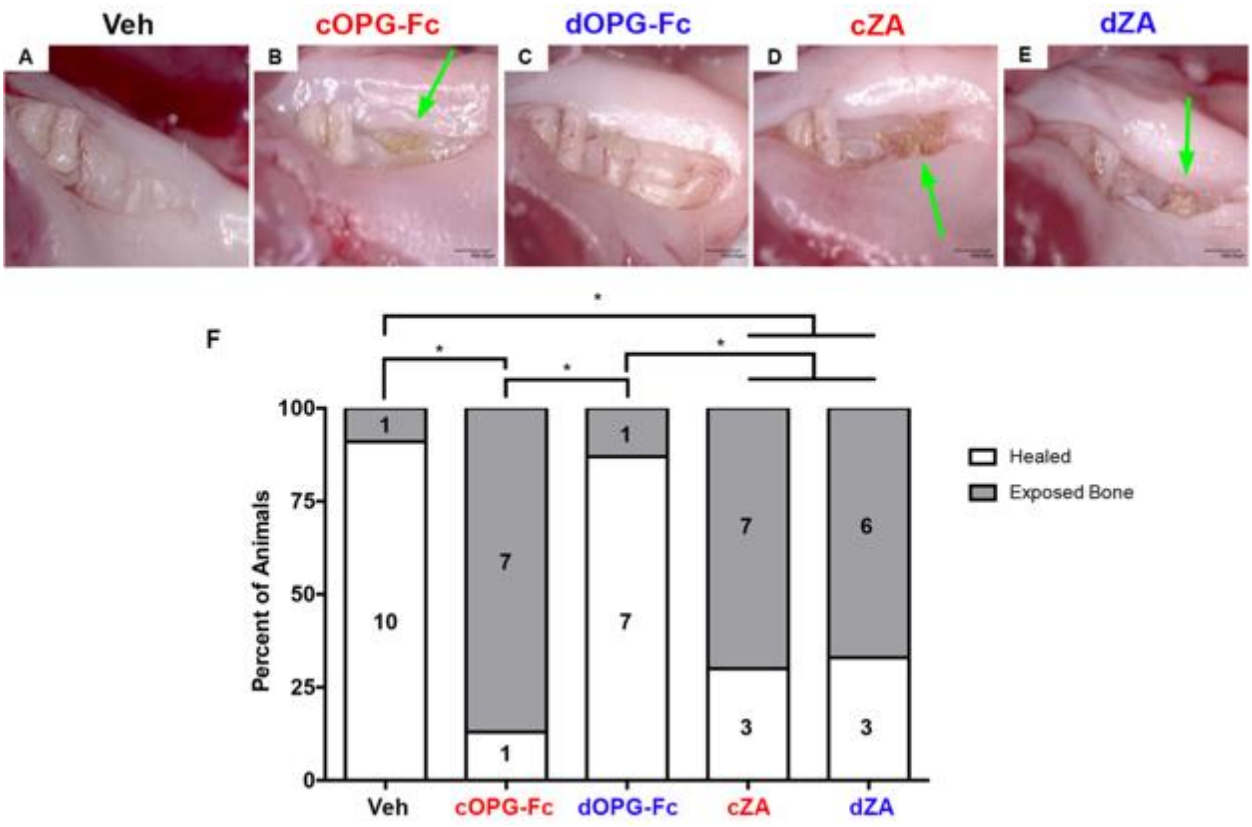

Figure 20: Clinical Findings in Animals with Discontinuation Before Tooth Extraction

Representative images of extraction sockets in Vehicle **(A)**, OPG-Fc **(B)**, dOPG-Fc **(C)**, ZA **(D)**, and dZA **(E)** animals. Green arrows point to areas of bone exposure. **(F)** Percent and number of animals with exposed bone or healed mucosa. Values in boxes represents number of animals with exposed bone. * = statistical significance $p<0.05$

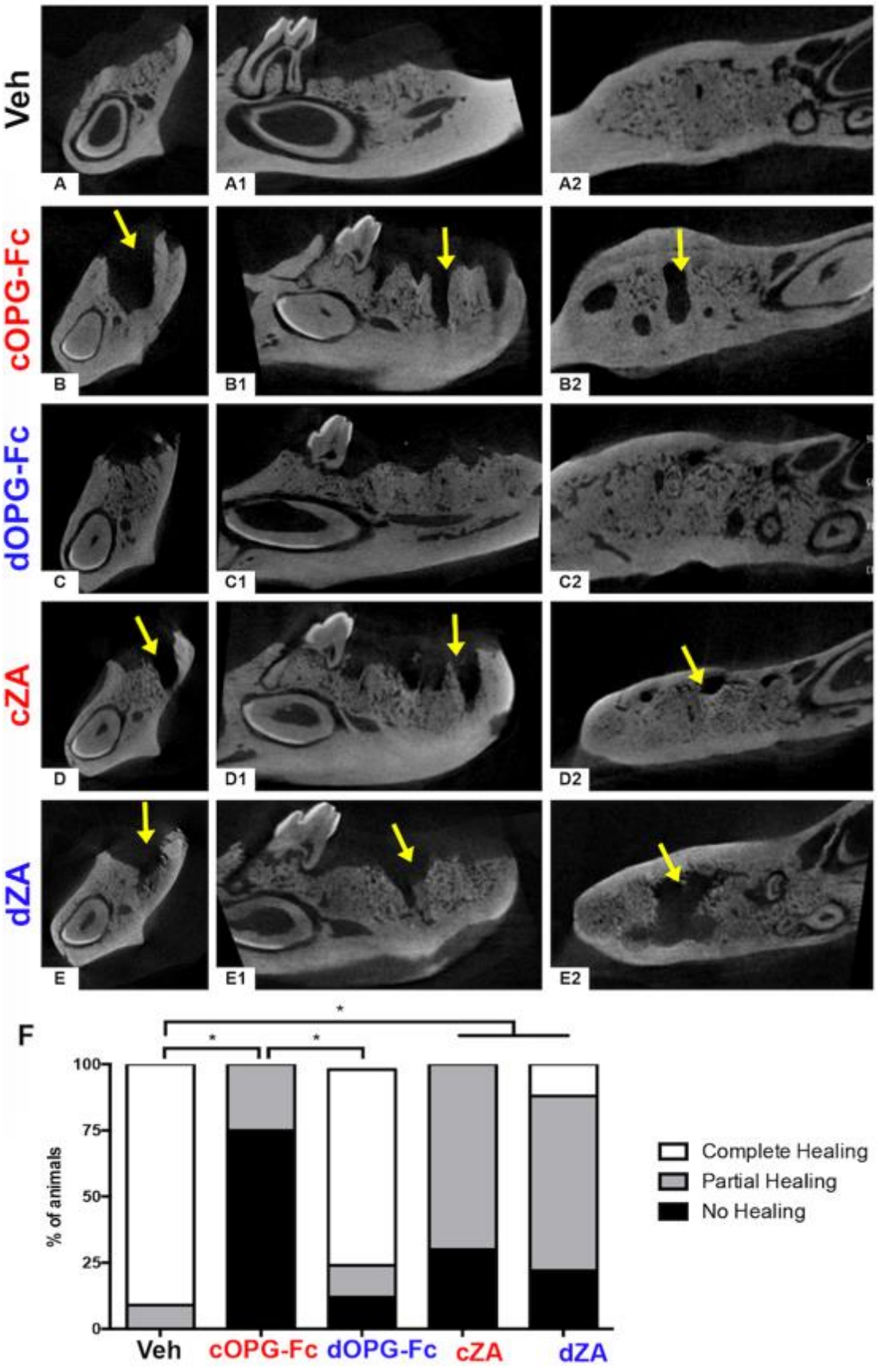
Veh
A
A1
A2
cOPG-Fc
B
B1
B2
dOPG-Fc
C
C1
C2
cZA
D
D1
D2
dZA
E
E1
E2
F
100
75
50
25
0
% of animals
Veh
cOPG-Fc
dOPG-Fc
cZA
dZA
Complete Healing
Partial Healing
No Healing

Figure 21: Radiographic Evaluation of Animals with Discontinuation Before Tooth Extraction

Representative µCT coronal **(A-E)**, sagittal **(A1-E1)**, and axial **(A2-E2)** sections of extraction sockets in Vehicle **(A)**, cOPG-Fc **(B)**, dOPG-Fc **(C)**, cZA **(D)**, and dZA **(E)** animals. Yellow arrows point to unhealed extraction sockets.

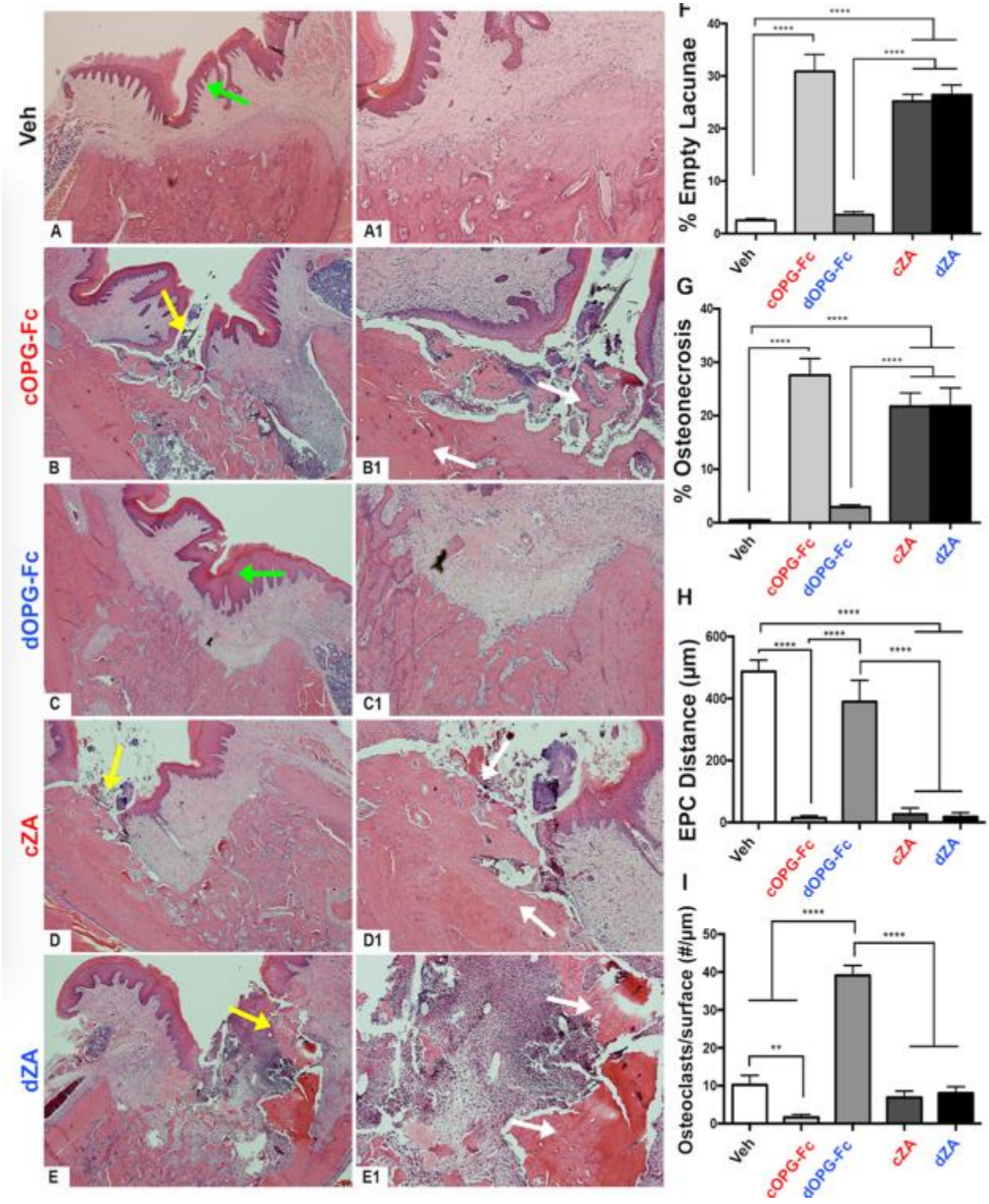
Veh
cOPG-Fc
dOPG-Fc
cZA
dZA
A
A1
B
B1
C
C1
D
D1
E
E1
F
% Empty Lacunae
G
% Osteonecrosis
H
EPC Distance (µm)
I
Osteoclasts/surface (#/µm)
Veh
cOPG-Fc
dOPG-Fc
cZA
dZA

Figure 22: Histologic Evaluation of Animals with Discontinuation Before Tooth Extraction

Low power (10x) and high power (20x) histologic microphotographs of Veh **(A-A1)**, cOPG-Fc **(B-B1)**, dOPG-Fc **(C-C1)**, cZA **(D-D1)**, and dZA **(E-E1)** groups. Quantification of **(F)** percent empty osteocytic lacunae, **(G)** percent osteonecrotic area, **(H)** epithelial to crest distance, and **(I)** osteoclasts per surface area. Yellow arrows point to epithelial discontinuity and bone exposure. White arrows point to areas of osteonecrosis. Data represents mean value ± SEM. **** = statistical significance $p<0.0001$, *** = statistical significance $p<0.001$, ** = statistical significance $p<0.01$, * = statistical significance $p<0.05$.

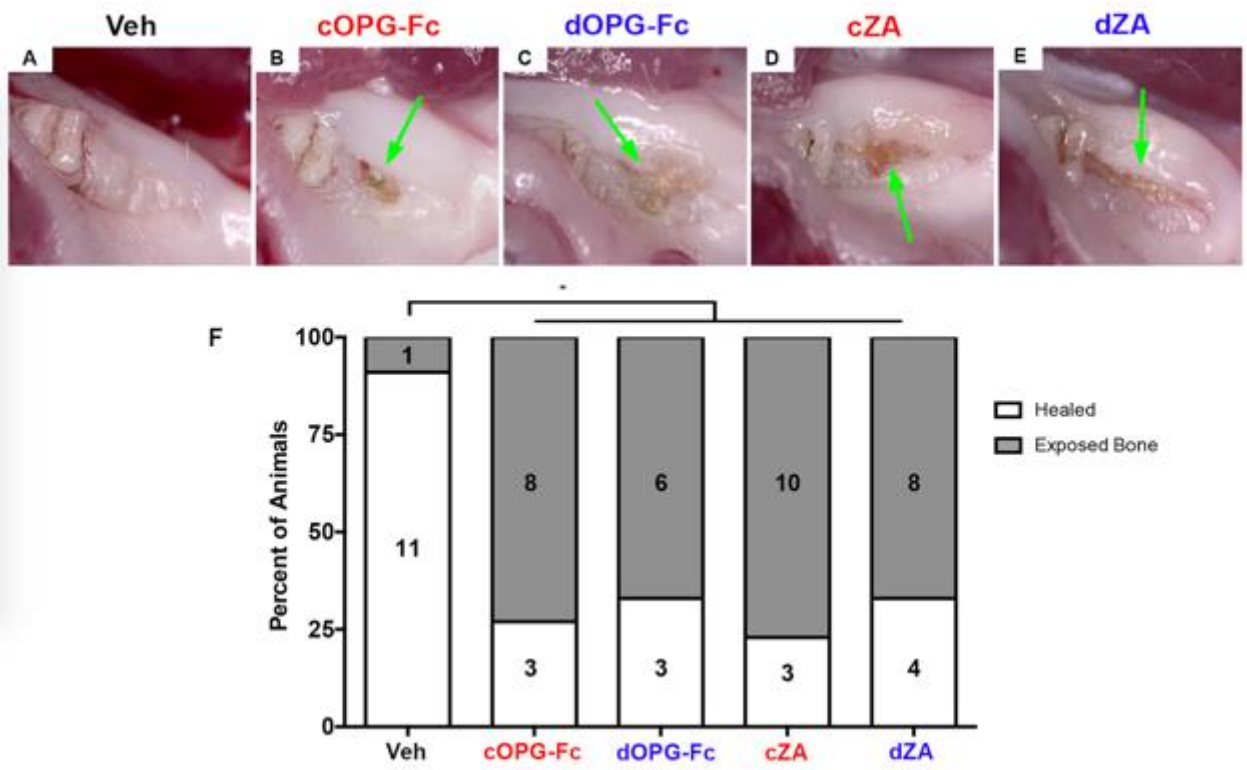

Figure 23: Clinical Findings in Animals with Discontinuation After Tooth Extraction

Representative images of extraction sockets in Vehicle **(A)**, OPG-Fc **(B)**, dOPG-Fc **(C)**, ZA **(D)**, and dZA **(E)** animals. Green arrows point to areas of bone exposure. **(F)** Percent and number of animals with exposed bone or healed mucosa. Values in boxes represents number of animals with exposed bone. * = statistical significance $p<0.05$

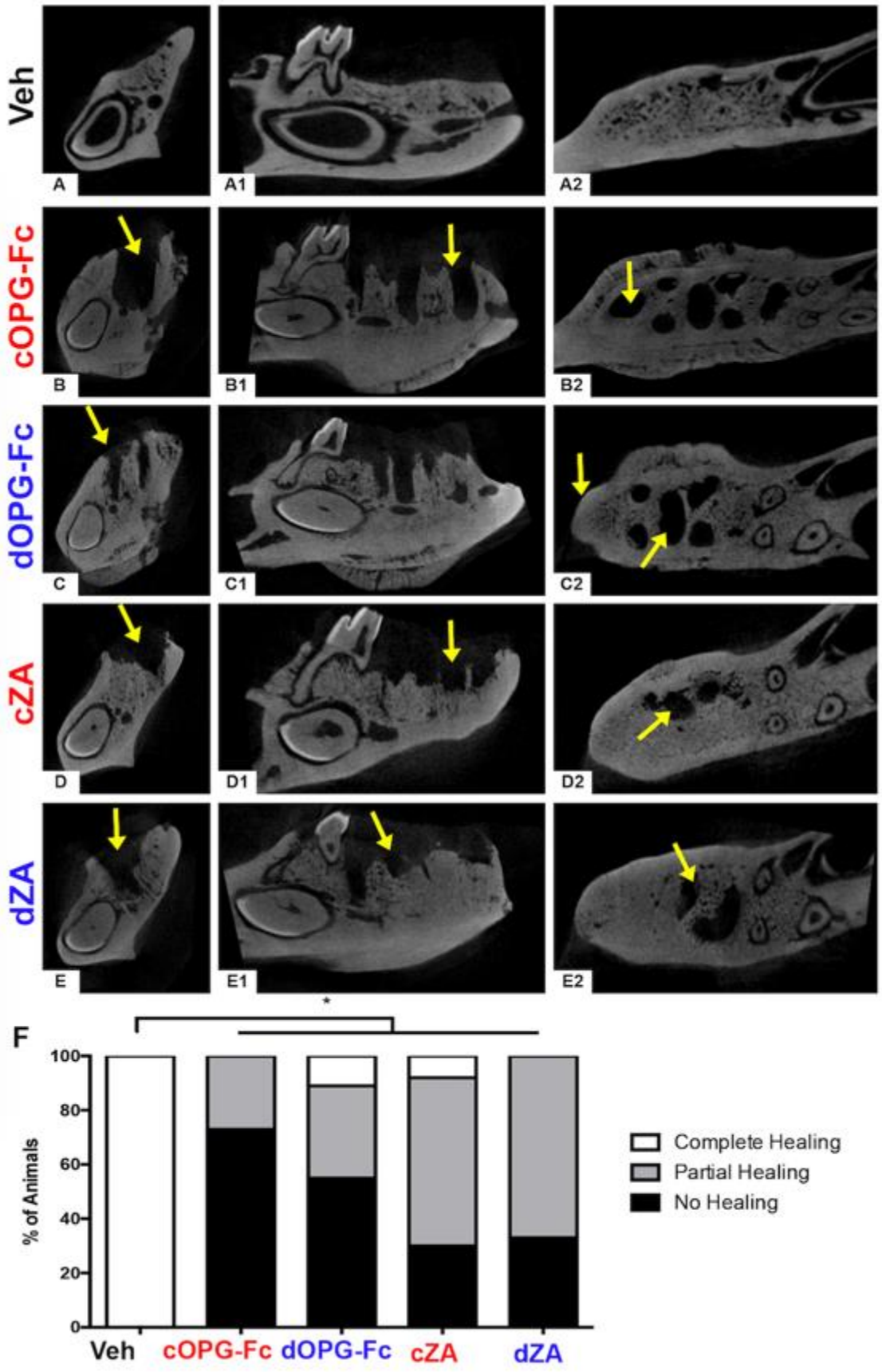
Veh
A
A1
A2
cOPG-Fc
B
B1
B2
dOPG-Fc
C
C1
C2
cZA
D
D1
D2
dZA
E
E1
E2
F
*
100
80
60
40
20
0
% of Animals
Veh
cOPG-Fc
dOPG-Fc
cZA
dZA
Complete Healing
Partial Healing
No Healing

Figure 24: Radiographic Evaluation of Animals with Discontinuation After Tooth Extraction

Representative µCT coronal **(A-E)**, sagittal **(A1-E1)**, and axial **(A2-E2)** images of socket healing in Vehicle **(A)**, cOPG-Fc **(B)**, dOPG-Fc **(C)**, cZA **(D)**, and dZA **(E)** animals. Yellow arrows point to unhealed extraction sockets.

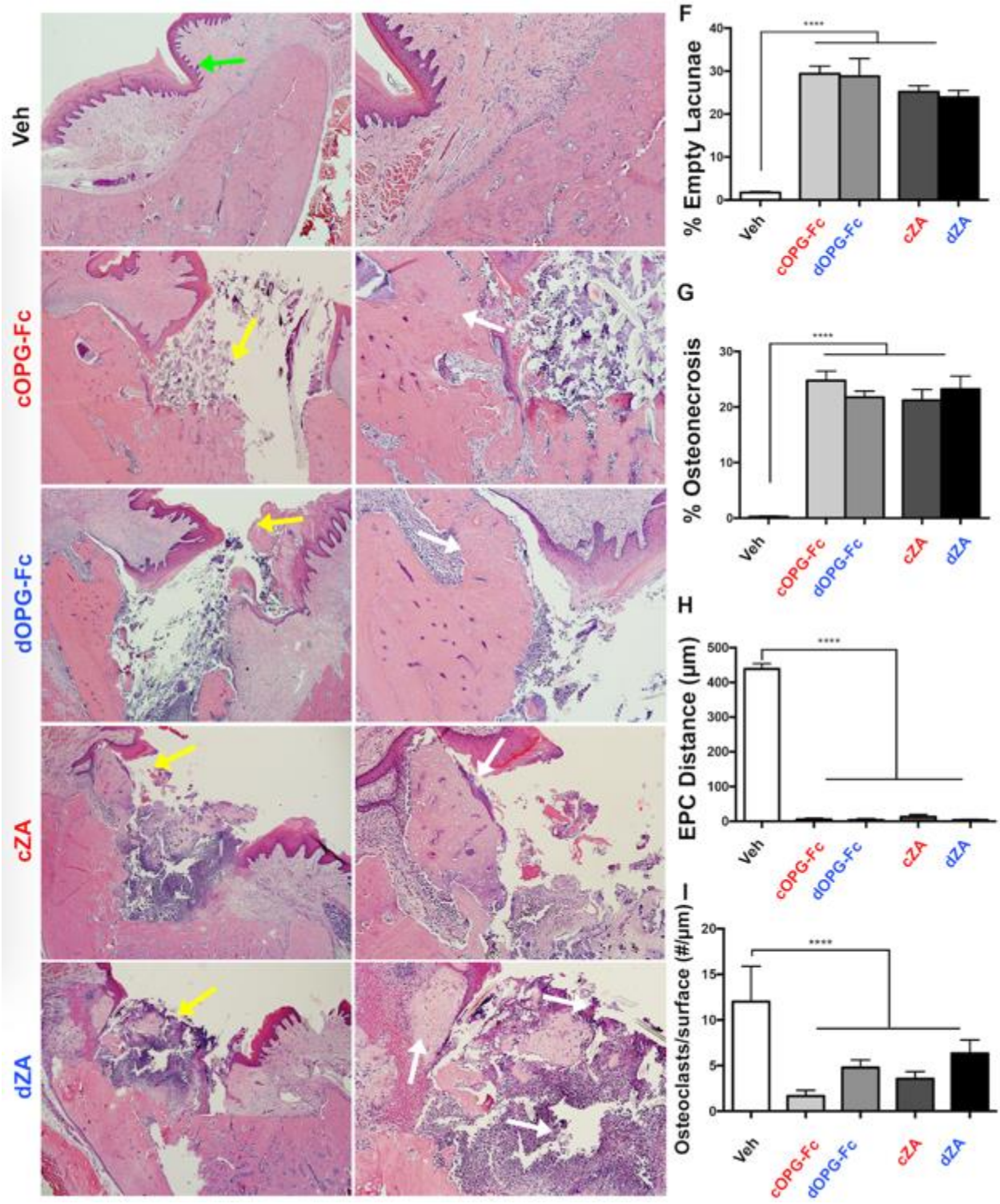
Veh
cOPG-Fc
dOPG-Fc
cZA
dZA
F
% Empty Lacunae
G
% Osteonecrosis
H
EPC Distance (µm)
Osteoclasts/surface (#/µm)
****
Veh
cOPG-Fc
dOPG-Fc
cZA
dZA

Figure 25: Histologic Evaluation of Animals with Discontinuation After Tooth Extraction

Low power (10x) and high power (20x) histologic microphotographs of Veh **(A-A1)**, cOPG-Fc **(B-B1)**, dOPG-Fc **(C-C1)**, cZA **(D-D1)**, and dZA **(E-E1)** groups. Quantification of **(F)** percent empty osteocytic lacunae, **(G)** percent osteonecrotic area, **(H)** epithelial to crest distance, and **(I)** osteoclasts per surface area. Yellow arrows point to epithelial discontinuity and bone exposure. White arrows point to areas of osteonecrosis. White arrows point to areas of osteonecrosis. Data represents mean value ± SEM. **** = statistical significance $p<0.0001$, *** = statistical significance $p<0.001$, ** = statistical significance $p<0.01$, * = statistical significance $p<0.05$.

Chapter 7: Conclusions

ONJ remains a side effect of anti-resorptive medications and has become exceedingly difficult to treat. Since ONJ's initial description in 2003 and 2004, much has been learned about its pathophysiology. It has become widely accepted that systemic anti-resorptive treatment, in conjunction with local events, such as tooth extraction, lead to ONJ development. It is also widely accepted, is that aggressive management of dental disease prior to anti-resorptive initiation can decrease risk of ONJ development. Therapeutic options remain scant, and are either surgical removal, or symptomology management.

Here, we have shown that periapical disease is necessary for ONJ development in rats treated with high-dose anti-resorptives. In non-treated animals, experimental periapical disease significantly increased the size of periapical lesions. Histologically, we observed bacterial colonization deep into the alveolar bone in ZA treated rats. ZA treated animals with extraction of teeth with experimental periapical developed clinically exposed bone. Radiographically, we observed an absence of socket healing, periosteal bone formation, and sequestration around areas of ONJ. Histologically, we observed empty osteocytic lacunae, with large areas of osteonecrosis. In contrast, non-treated animals healed uneventfully, as did ZA animals with extraction of healthy teeth. These findings point to the importance of inflammation and infection in ONJ development.

Other treatments for osteoporosis have also been developed, including antibodies against the osteocyte secreted protein, sclerostin. Romosozumab, a sclerostin antibody, has largely anabolic actions; however, in clinical trials, patients also had decreased levels of bone resorption markers. Thus, these patients could also develop ONJ. We evaluated ovariectomized rats treated with Scl-Ab, ZA, and control rats for ONJ development using a rat model of experimental

periodontitis. Animals treated with ZA developed histologic and radiographic signs of ONJ, with areas of osteonecrosis and exposed bone. In contrast, control animals, and Scl-Ab treated animals did not develop areas of ONJ. It appears that the local inflammatory environment caused by experimental periodontitis overcomes the systemic catabolic effect of anti-resorptives.

We chose to expand our efforts to clinical studies, in an attempt to correlate infection and inflammation to ONJ. A large cohort of patients was treated using local wound care, a treatment option that focuses on decreasing inflammation and infection around areas of exposed bone. Interestingly, most patients treated with this wound care regimen would go on to sequester their exposed, necrotic bone, leaving fully healed underlying mucosa. To further examine our cohort of patients, we evaluated their wound care scores, defined as the presence of infection and edema. Patients who had better wound care scores, indicating that their lesions were absence of inflammation and plaque, healed at a quicker rate than those with poor wound care. Collectively, this data also points to the notion that infection and inflammation are crucial in ONJ development and pathogenesis.

Genetic diseases can often provide insight on disease processes. Two genetic conditions, osteopetrosis, and pycnodysostosis create an anti-resorptive like phenotype. However, these genetic conditions are exceedingly rare. As disruption of osteoclast function is a main hypothesis in ONJ, we evaluated a set of patients with osteopetrosis and pycnodysostosis who had clinical features similar to that of ONJ. We evaluated clinic, radiographic, and histologic features of these patients and observed a striking similarity to patients with ONJ. Interestingly, the patients developed exposed bone mostly following tooth extraction or dental disease, similar to patients with ONJ. We thus, believe that these patients from a genetic form of ONJ. These findings prove that a dysfunctional osteoclast is central in ONJ development.

Finally, we explored potential therapeutic options patients on anti-resorptives, and for patients with established ONJ. Clinical translation of these studies is exceedingly important; we thus chose two specific events. The first study evaluated whether discontinuation of BPs vs RANKL inhibitors prior to tooth extraction would ameliorate ONJ development. Using our previously described model of ONJ, we observed that discontinuation of RANKL inhibitors, but not BPs, prior to tooth extraction ameliorated ONJ development. Using this rodent model, we tested whether discontinuation of anti-resorptives in cases established ONJ would accelerate healing. Interestingly, discontinuation of neither agent lead to any disease resolution. These findings are important because they provide first steps to the use of drug holidays in a patient population.

Future Directions

Our studies here have identified the importance bacterial inflammation and infection and osteoclast dysfunction in ONJ pathogenesis. We have also identified that anti-resorptive discontinuation can be a potential preventative measure in patients prior to tooth extraction.

Our future studies will now use our well described animal model to evaluate ONJ development in mice treated with a potent Cathepsin K inhibitor. Cathepsin K is a lysosomal protease important for the degradation of bone matrix. We observed ONJ in patients with the genetic disease pycnodysostosis, which renders osteoclast dysfunctional due to nonfunctional cathepsin K. Interestingly, Cathepsin K was once a target for osteoporosis treatment, as it was thought to potentially have the same effects as BPs without the side effects, as it would not lead to complete abolition of osteoclasts. We will treat animals with high dose systemic cathepsin K, and induce ONJ using well established models of experimental periodontitis in the maxilla, and tooth extraction following induction of experimental periapical disease in the mandible. If we observe ONJ in animals treated with cathepsin K inhibitors, we will confirm that osteoclast dysfunction is crucial to ONJ development.

Our animal studies have identified that discontinuation of neither BPs, nor denosumab helps lead to disease resolution in rodents with ONJ. We believe that these findings are important; however, our model was limited by a short time period to allow for clinical healing. Importantly, we believe that a longer period of drug cessation or later time-point for evaluation could potentially lead to ONJ resolution in these rodents. We will thus repeat this experiment with a longer time period, allowing for at least 3 half-lives to remove most of the circulating denosumab.

Finally, while our studies have identified the importance of osteoclast dysfunction and infection/inflammation, we are now focused on finding tissue level data in ONJ pathogenesis, allowing for development of reproducible models, we now will utilize these models to evaluate early events in ONJ pathogenesis. We will collect RNA from the gingival tissue and from the alveolar crest around mice with EP. We will collect tissue from untreated mice, mice treated with OPG-Fc, and mice treated with BP. After collection, we will evaluate differential gene expression, targeting inflammatory and necrotic pathways.

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